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(54) Title: INDUCTION OF IMMUNITY AGAINST TUMOR SELF-ANTIGENS (57) Abstract This invention provides methods and compositions for breaking tolerance to a self-antigen, especially in the context of a tumor-associated antigen. In one embodiment, the method utilized altered tumor antigens or tumor antigens derived from heterologous species to break immunological tolerance and induce a cross-reactive immune response against the corresponding native or self-antigen.		

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INDUCTION OF IMMUNITY AGAINST TUMOR SELF-ANTIGENS

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Application No. 60/078,889, filed March 20, 1998, the contents of which are hereby incorporated by reference.

TECHNICAL FIELD

10 This invention is in the field of molecular immunology and medicine. In particular, the present invention provides compositions and methods for inducing an immune response to a native self-antigen in a subject.

BACKGROUND

15 The goal of vaccination is to generate a protective immune response and an expanded population of memory cells ready to encounter an agent identified as foreign, which will then elicit a potent secondary immune response. T and B cells are highly antigen specific and can develop into memory cells, and therefore are the target for a successful vaccine.

20 Tumor specific T cells, derived from cancer patients, will bind and lyse tumor cells. This specificity is based on their ability to recognize short amino acid sequences (epitopes) presented on the surface of the tumor cells by MHC class I and class II molecules. These epitopes are derived from the proteolytic degradation of intracellular proteins called tumor antigens encoded by genes that
25 are either uniquely or aberrantly expressed in tumor or cancer cells.

 The availability of specific anti-tumor T cells has enabled the identification of tumor antigens and subsequently the generation of cancer vaccines designed to provoke an anti-tumor immune response. A critical target of vaccines is the specialized antigen-presenting cell ("APC"), the most

immunologically powerful of which is the bone marrow-derived dendritic cell ("DC").

DCs are potent antigen presenters that express high levels of co-stimulatory molecules and are capable of activating both CD4⁺ and CD8⁺ naive T lymphocytes. Results obtained in several animal models have shown that DCs pulsed with defined tumor-associated peptides or with peptides eluted from the surface of tumor cells are capable of inducing an antigen-specific CTL response resulting in protection from tumor challenge and, in some instances, regression of established tumors. The same type of approach has also been tested in human clinical trials with encouraging results. For example, Hsu et al. have reported that four B cell lymphoma patients infused with autologous DCs pulsed with tumor-specific idiotype protein all developed an idiotype-specific proliferative response accompanied by complete tumor regression in two patients and partial regression in a third. Hsu et al. (1996) *Nature Med.* 2:52. More recently, Nestle et al. reported that melanoma patients treated with autologous DCs pulsed with tumor lysate or a cocktail of CTL peptide epitopes, developed cell-mediated immunity with objective clinical responses in 5 out of 16 patients evaluated. Nestle et al. (1998) *Nature Med.* 4:328.

Successful cancer therapy, similar to the ones noted above, is rare. The high incidence of failure may be due to the fact that naturally occurring neoplasms do not possess antigens that can serve as inducers and/or targets for a tumor destructive immune response, although immunological reactions mediated by either lymphocytes or antibodies to cultivated human tumors have been reported. Hellstrom K. and Hellstrom I. (1969) *Adv. Cancer Res.* 12:167. Indeed, mechanisms of systemic immune tolerance to self have begun to emerge, particularly from studies in transgenic mouse systems. Hanahan D. (1990) *Ann. Rev. Cell Biol.* 6:493. Mechanisms of systemic immune tolerance include deletion of potentially autoreactive B or T cells, induction of anergy in B and T cells, and the poorly defined phenomenon of suppression of immune response by suppressor cells. Houghton and Lewis, pages 37-54 in Forni, et al., eds. (1994) *CYTOKINE-INDUCED TUMOR IMMUNOGENICITY*, Academic Press, New York.

Thus, a need exists to overcome immune tolerance to self-antigens and to provide an effective cancer vaccine. This invention satisfies these needs and provides related advantages as well.

5

DISCLOSURE OF THE INVENTION

In the present invention, immunization is carried out with a heterologous antigen or an altered antigen that is structurally distinct from the self-antigen or “native” antigen yet is still capable of inducing an immune response against the self-antigen. Such antigens are immunogenic (seen as foreign) and serve to
10 induce an immune response that cross-reacts with the native antigen.

In the context of cancer gene therapy, the invention comprises using modified (altered) tumor antigens or tumor antigens derived from heterologous species to break immunological tolerance and induce a cross-reactive immune response against the corresponding native or self-antigen. For example,
15 immunizing humans against the human melanoma antigen gp100 requires breaking tolerance against a self-antigen. As shown below, the use of the non self-antigen can provide protective immunity and tumor reduction *in vivo*. Immunization and therapy are accomplished by any of the following methods: 1) administration of a vector encoding altered tumor antigen or antigen from a
20 heterologous species; 2) infecting dendritic cells *ex vivo* or *in vivo* with the same vector; or 3) use of transduced dendritic cells or APCs to stimulate production of an enriched population of antigen-specific immune effector cells that can be adoptively transferred into the host.

Antigen presenting cells such as dendritic cells also are useful to expand a
25 population of immune effector cells that specifically recognize and lyse the cells presenting the heterologous antigen and its native or self-counterpart. The expanded immune effector cell populations and their use in prophylactical and therapeutical methods also are provided herein.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A through 1D show the results of immunizing mice with syngeneic DCs. Five female C57BL/6 mice (represented by five different symbols in the panels) were immunized with B16 melanoma using dendritic cells transfected with Ad vector encoding homologous mouse gp100 versus heterologous human gp100.

Figures 2A through 2C show induction of CTL activity following immunization with Ad2/hugp100vl vector or Ad2/hugp100vl-transduced DCs. Spleens from groups of 3 animals were collected 15 days after i.v. administration of vehicle (Figure 2A), Ad2/hugp100vl-transduced DCs (Figure 2B) or i.d. delivery of Ad2/hugp100vl vector (Figure 2C). Pooled spleen cells from each group were re-stimulated *in vitro* with syngeneic SVB6KHA fibroblasts transduced with Ad2/hugp100v 1 and were tested for cytolytic activity after 6 days of culture. Targets consisted of B16 cells and SVB6KHA fibroblasts untransduced or transduced with Ad2/hugp100v 1 or wild type Ad2 deleted for E3 (SVB6KHA-Ad2Δ2.9). Figure legend: (-●- B16; -■- SVB6KHA - Untransduced; -▲- SVB6KHA-Ad2/hugp100v1; -◆- SVB6HA-Ad2Δ2.9).

Figure 3 compares the effectiveness of immunization with DCs transduced with Ad vector encoding various melanoma-associated antigens. The figure shows the evaluation of the nature of the antigen. Figure legend: (-□- Untransduced DCs; -◇- Ad2/hugp100v1 DCs; -o- Ad2mgp100 DCs; -Δ- Ad2/mTRP-2 DCs). Groups of 5 C57BL/6 mice were injected i.v. with 5×10^5 DCs that were either untransduced or transduced with Ad2/hugp100v1, Ad2/mgp100 or Ad2/mTRP-2 vector. The animals were challenged 15 days later with a s.c. injection of 2×10^4 B16 melanoma cells.

Figure 4 shows the frequency of gp100-reactive splenic T lymphocytes following immunization with Ad2/hugp100- or Ad2/empty vector-transduced DCs. Spleen cells from immunized mice were stimulated *in vitro* with a cytotoxic T lymphocyte peptide epitope derived from hugp100 (open bar); or the corresponding epitope from mgp100 (solid bar). An ovalbumin-derived epitope

was used as a negative control (hatched bar). The number of T lymphocytes that produced γ -interferon upon recognition of peptide was measured by elispot.

MODES FOR CARRYING OUT THE INVENTION

Throughout this disclosure, various publications, patents and published patent specifications are referenced by an identifying citation. The disclosures of these publications, patents and published patent specifications are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

Definitions

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of immunology, molecular biology, microbiology, cell biology and recombinant DNA, which are within the skill of the art. See, *e.g.*, Sambrook, Fritsch and Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel, et al. eds., (1987)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.): PCR 2: A PRACTICAL APPROACH (M.J. MacPherson, B.D. Hames and G.R. Taylor eds. (1995)), Harlow and Lane, eds. (1989) ANTIBODIES, A LABORATORY MANUAL, and ANIMAL CELL CULTURE (R.I. Freshney, ed. (1987)).

As used herein, certain terms may have the following defined meanings.

As used in the specification and claims, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

The term "genetically modified" means containing and/or expressing a foreign gene or nucleic acid sequence which in turn, modifies the genotype or phenotype of the cell or its progeny. In other words, it refers to any addition, deletion or disruption to a cell's endogenous nucleotides.

As used herein, the term "cytokine" refers to any one of the numerous factors that exert a variety of effects on cells, for example, inducing growth or

proliferation. Non-limiting examples of cytokines which may be used alone or in combination in the practice of the present invention include, interleukin-2 (IL-2), stem cell factor (SCF), interleukin 3 (IL-3), interleukin 6 (IL-6), interleukin 12 (IL-12), G-CSF, granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-1 alpha (IL-1 α), interleukin-11 (IL-11), MIP-1 α , leukemia inhibitory factor (LIF), c-kit ligand, thrombopoietin (TPO) and flt3 ligand. The present invention also includes culture conditions in which one or more cytokine is specifically excluded from the medium. Cytokines are commercially available from several vendors such as, for example, Genzyme (Framingham, MA), Genentech (South San Francisco, CA), Amgen (Thousand Oaks, CA), R&D Systems and Immunex (Seattle, WA). It is intended, although not always explicitly stated, that molecules having similar biological activity as wild-type or purified cytokines (e.g., recombinantly produced or muteins thereof) are intended to be used within the spirit and scope of the invention.

The term "antigen presenting cell" ("APC"), as used herein, intends any cell which presents on its surface an antigen in association with a major histocompatibility complex molecule, or portion thereof, or, alternatively, one or more non-classical MHC molecules, or a portion thereof. Examples of suitable APCs are discussed in detail below and include, but are not limited to, whole cells such as macrophages, dendritic cells, B cells, hybrid APCs, and foster antigen presenting cells or other cell type(s) expressing the necessary MHC and co-stimulatory molecules. Methods of making hybrid APCs have been described. See, for example, International Patent Application Publication Nos. WO 98/46785 and WO 95/16775.

Dendritic cells (DCs) are potent antigen-presenting cells. It has been shown that DCs provide all the signals required for T cell activation and proliferation. These signals can be categorized into two types. The first type, which gives specificity to the immune response, is mediated through interaction between the T-cell receptor/CD3 ("TCR/CD3") complex and an antigenic peptide presented by a major histocompatibility complex ("MHC") class I or II protein on the surface of APCs. This interaction is necessary, but not sufficient, for T cell

activation to occur. In fact, without the second type of signals, the first type of signals can result in T cell anergy. The second type of signals, called co-stimulatory signals, is neither antigen-specific nor MHC-restricted, and can lead to a full proliferation response of T cells and induction of T cell effector functions in the presence of the first type of signals. As used herein, "dendritic cell" is to include, but not be limited to a pulsed dendritic cell, a foster cell, a dendritic cell hybrid or a genetically modified dendritic cell. Methods for generating dendritic cells from peripheral blood or bone marrow progenitors have been described (Inaba et al. (1992) *J. Exp. Med.* **175**:1157; Inaba et al. (1992) *J. Exp. Med.* **176**:1693-1702; Romani et al. (1994) *J. Exp. Med.* **180**:83-93; Sallusto et al. (1994) *J. Exp. Med.* **179**:1109-1118; Bender et al. (1996) *J. Imm. Methods* **196**:121-135; and Romani et al. (1996) *J. Imm. Methods* **196**:137-151).

"Co-stimulatory molecules" are molecules involved in the interaction between receptor-ligand pairs expressed on the surface of antigen presenting cells and T cells. "Co-stimulatory activity" was originally defined as an activity provided by bone-marrow-derived accessory cells such as macrophages and dendritic cells, the so called "professional" APCs. Several molecules have been shown to enhance co-stimulatory activity. These are heat stable antigen (HSA) (Liu Y. et al. (1992) *J. Exp. Med.* **175**:437), chondroitin sulfate-modified MHC invariant chain (Ii-CS) (Naujokas M.F. et al. (1993) *Cell* **74**:257), intracellular adhesion molecule 1 (ICAM-1) (Van Seventer G.A. (1990) *J. Immunol.* **144**:4579), B7-1 and B7-2/B70 (Schwartz R.H. (1992) *Cell* **71**:1065) and B7's counter-receptor CD28 or CTLA-4 on T cells (Freeman et al. (1993) *Science* **262**:909; Young et al. (1992) *J. Clin. Invest.* **90**: 229; and Nabavi et al. (1992) *Nature* **360**:266). Other important co-stimulatory molecules are CD40, CD54, CD80, CD86. As used herein, the term "co-stimulatory molecule" encompasses any single molecule or combination of molecules which, when acting together with a peptide/MHC complex bound by a TCR on the surface of a T cell, provides a co-stimulatory effect which achieves activation of the T cell that binds the peptide. The term thus encompasses B7, or other co-stimulatory molecule(s) on an antigen-presenting matrix such as an APC, fragments thereof (alone,

complexed with another molecule(s), or as part of a fusion protein) which, together with peptide/MHC complex, binds to a cognate ligand and results in activation of the T cell when the TCR on the surface of the T cell specifically binds the peptide. Co-stimulatory molecules are commercially available from a variety of sources, including, for example, Beckman Coulter. It is intended, although not always explicitly stated, that molecules having similar biological activity as wild-type or purified co-stimulatory molecules (e.g., recombinantly produced or muteins thereof) are intended to be used within the spirit and scope of the invention.

The term "antigen" is used in its broadest sense and includes minimal epitopes and chimeric molecules in addition to isolated full length proteins. A "self-antigen", also referred to herein as a "native antigen", is an antigenic peptide that induces little or no immune response in the subject due to self-tolerance to the antigen. An example of a self-antigen is the melanoma antigen gp100. The antigen of this vaccine is "heterologous" (i.e., allogeneic or a homologue from an isolated species, e.g., a murine antigen administered to a human patient) or an "altered antigen" as compared to the corresponding native self-antigen. The heterologous or altered antigen also can be made by chemical synthesis.

The term "immune effector cells" refers to cells capable of binding an antigen or which mediate an immune response. These cells include, but are not limited to, T cells, B cells, monocytes, macrophages, NK cells and cytotoxic T lymphocytes (CTLs), for example CTL lines, CTL clones, and CTLs from tumor, inflammatory, or other infiltrates. Certain diseased tissues express specific antigens and CTLs specific for these antigens have been identified. For example, approximately 80% of melanomas express the antigen known as gp100.

The term "immune effector molecule" as used herein, refers to molecules capable of antigen-specific binding, and includes antibodies, T cell antigen receptors, and MHC Class I and Class II molecules.

A "naïve" immune effector cell is an immune effector cell that has never been exposed to an antigen.

As used herein, the term “educated, antigen-specific immune effector cell” is an immune effector cell as defined above, which has encountered antigen and which is specific for that antigen. An educated, antigen-specific immune effector cell may be activated upon binding antigen. “Activated” implies that the cell is no longer in G₀ phase, and begins to produce cytokines characteristic of the cell type. For example, activated CD4⁺ T cells secrete IL-2 and have a higher number of high affinity IL-2 receptors on their cell surfaces relative to resting CD4⁺ T cells.

A peptide or polypeptide of the invention may be preferentially recognized by antigen-specific immune effector cells, such as B cells and T cells. In the context of T cells, the term “recognized” intends that a peptide or polypeptide of the invention, comprising one or more synthetic antigenic epitopes, is recognized, i.e., is presented on the surface of an APC together with (i.e., bound to) an MHC molecule in such a way that a T cell antigen receptor (TCR) on the surface of an antigen-specific T cell binds to the epitope wherein such binding results in activation of the T cell. The term “preferentially recognized” intends that a polypeptide of the invention is substantially recognized, as defined above, by a T cell specific for an antigen. Assays for determining whether an epitope is recognized by an antigen-specific T cell are known in the art and are described herein.

The term “syngeneic” or “autologous” as used herein, indicates the origin of a cell. Thus, a cell being administered to an individual (the “recipient”) is autogeneic if the cell was derived from that individual (the “donor”) or a genetically identical individual. An syngeneic cell can also be a progeny of an syngeneic cell. The term also indicates that cells of different cell types are derived from the same donor or genetically identical donors. Thus, an effector cell and an antigen presenting cell are said to be syngeneic if they were derived from the same donor or from an individual genetically identical to the donor, or if they are progeny of cells derived from the same donor or from an individual genetically identical to the donor.

Similarly, the term “allogeneic” as used herein, indicates the origin of a cell. Thus, a cell being administered to individual (the “recipient”) is allogeneic if

the cell was derived from an individual not genetically identical to the recipient; in particular, the term relates to non-identity in expressed MHC molecules. An allogeneic cell can also be a progeny of an allogeneic cell. The term also indicates that cells of different cell types are derived from genetically non-identical donors, or if they are progeny of cells derived from genetically non-identical donors. For example, an APC is said to be allogeneic to an effector cell if they are derived from genetically non-identical donors.

A "hybrid" cell refers to a cell having both antigen presenting capability and also expresses one or more specific antigens. In one embodiment, these hybrid cells are formed by fusing, *in vitro*, APCs with cells that are known to express the one or more antigens of interest.

The term "culturing" refers to the *in vitro* propagation of cells or organisms on or in media of various kinds. It is understood that the descendants of a cell grown in culture may not be completely identical (either morphologically, genetically, or phenotypically) to the parent cell. By "expanded" is meant any proliferation or division of cells.

A "subject" is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, murines, simians, humans, farm animals, sport animals, and pets.

As used herein, "expression" refers to the process by which polynucleotides are transcribed into mRNA and translated into peptides, polypeptides, or proteins. If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA, if an appropriate eukaryotic host is selected. Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the *lac* promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG (Sambrook et al. (1989) *Supra*). Similarly, an eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors can be

obtained commercially or assembled by the sequences described in methods well known in the art, for example, the methods described below for constructing vectors in general.

5 The terms "major histocompatibility complex" or "MHC" refers to a complex of genes encoding cell-surface molecules that are required for antigen presentation to T cells and for rapid graft rejection. In humans, the MHC complex is also known as the HLA complex. The proteins encoded by the MHC complex are known as "MHC molecules" and are classified into class I and class II MHC molecules. Class I MHC molecules include membrane heterodimeric proteins
10 made up of an α chain encoded in the MHC associated noncovalently with β 2-microglobulin. Class I MHC molecules are expressed by nearly all nucleated cells and have been shown to function in antigen presentation to $CD8^+$ T cells. Class I molecules include HLA-A, -B, and -C in humans. Class II MHC molecules also include membrane heterodimeric proteins consisting of
15 noncovalently associated α and β chains. Class II MHC are known to participate in antigen presentation to $CD4^+$ T cells and, in humans, include HLA-DP, -DQ, and DR. The term "MHC restriction" refers to a characteristic of T cells that permits them to recognize antigen only after it is processed and the resulting antigenic peptides are displayed in association with either a self class I or class II
20 MHC molecule. Methods of identifying and comparing MHC are well known in the art and are described in Allen M. et al. (1994) Human Imm. 40:25; Santamaria P. et al. (1993) Human Imm. 37:39 and Hurley C.K. et al. (1997) Tissue Antigens 50:401.

25 The term "peptide" is used in its broadest sense to refer to a compound of two or more subunit amino acids, amino acid analogs, or peptidomimetics. The subunits may be linked by peptide bonds. In another embodiment, the subunit may be linked by other bonds, *e.g.* ester, ether, etc. As used herein the term "amino acid" refers to either natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs and
30 peptidomimetics. A peptide of three or more amino acids is commonly called an

oligopeptide if the peptide chain is short. If the peptide chain is long, the peptide is commonly called a polypeptide or a protein.

A "composition" is intended to mean a combination of active agent and another compound or composition, inert (for example, a solid support, a detectable agent or label) or active, such as an adjuvant.

As used herein, "solid phase support" or "solid support" used interchangeably, is not limited to a specific type of support. Rather a large number of supports are available and are known to one of ordinary skill in the art. Solid phase supports include silica gels, resins, derivatized plastic films, glass beads, cotton, plastic beads, and alumina gels. As used herein, "solid support" also includes synthetic antigen-presenting matrices, cells, and liposomes. A suitable solid phase support may be selected on the basis of desired end use and suitability for various protocols. For example, for peptide synthesis, solid phase support may refer to resins such as polystyrene (*e.g.*, PAM-resin obtained from Bachem Inc., Peninsula Laboratories, etc.), POLYHIPE® resin (obtained from Aminotech, Canada), polyamide resin (obtained from Peninsula Laboratories), polystyrene resin grafted with polyethylene glycol (TentaGel®, Rapp Polymere, Tubingen, Germany) or polydimethylacrylamide resin (obtained from Milligen/Bioscience, California).

A "pharmaceutical composition" is intended to include the combination of an active agent with a carrier, inert or active, making the composition suitable for diagnostic or therapeutic use *in vitro*, *in vivo* or *ex vivo*.

As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, see Martin, REMINGTON'S PHARM. SCI., 15th Ed. (Mack Publ. Co., Easton (1975)).

An "effective amount" is an amount sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations, applications or dosages.

The term "immunomodulatory agent" as used herein, is a molecule, a macromolecular complex, or a cell that modulates an immune response and encompasses a synthetic antigenic peptide of the invention alone or in any of a variety of formulations described herein; a polypeptide comprising a synthetic antigenic peptide of the invention; a polynucleotide encoding a peptide or polypeptide of the invention; a synthetic antigenic peptide of the invention bound to a Class I or a Class II MHC molecule on an antigen-presenting matrix, including an APC and a synthetic antigen-presenting matrix (in the presence or absence of co-stimulatory molecule(s)); a synthetic antigenic peptide of the invention covalently or non-covalently complexed to another molecule(s) or macromolecular structure; and an educated, antigen-specific immune effector cell which is specific for a peptide of the invention.

The term "modulate an immune response" includes inducing (increasing, eliciting) an immune response; and reducing (suppressing) an immune response. An immunomodulatory method (or protocol) is one that modulates an immune response in a subject.

As used herein, the term "inducing an immune response in a subject" is a term well understood in the art and intends that an increase of at least about 2-fold, more preferably at least about 5-fold, more preferably at least about 10-fold, more preferably at least about 100-fold, even more preferably at least about 500-fold, even more preferably at least about 1000-fold or more in an immune response to an antigen (or epitope) can be detected (measured), after introducing the antigen (or epitope) into the subject, relative to the immune response (if any) before introduction of the antigen (or epitope) into the subject. An immune response to an antigen (or epitope), includes, but is not limited to, production of an antigen-specific (or epitope-specific) antibody, and production of an immune cell expressing on its surface a molecule which specifically binds to an antigen (or epitope). Methods of determining whether an immune response to a given antigen (or epitope) has been induced are well known in the art. For example, antigen-specific antibody can be detected using any of a variety of immunoassays known in the art, including, but not limited to, ELISA, wherein, for example, binding of

an antibody in a sample to an immobilized antigen (or epitope) is detected with a detectably-labeled second antibody (e.g., enzyme-labeled mouse anti-human Ig antibody). Immune effector cells specific for the antigen can be detected any of a variety of assays known to those skilled in the art, including, but not limited to, ⁵¹Cr-release assays, ³H-thymidine uptake assays or induction of cytokine release.

As used herein, the term "a disease or condition related to a population of CD4⁺ or CD8⁺ T cells" is one which can be related to a population of CD4⁺ or CD8⁺ T cells, such that these cells are primarily responsible for the pathogenesis of the disease; it is also one in which the presence of CD4⁺ or CD8⁺ T cells is an indicia of a disease state; it is also one in which the presence of a population CD4⁺ or CD8⁺ T cells is not the primary cause of the disease, but which plays a key role in the pathogenesis of the disease; it is also one in which a population of CD4⁺ or CD8⁺ T cells mediates an undesired rejection of a foreign antigen. Examples of a condition related to a population of CD4⁺ or CD8⁺ T cells include, but are not limited to, autoimmune disorders, graft rejection, immunoregulatory disorders, and anaphylactic disorders.

As used herein, the terms "neoplastic cells", "neoplasia", "tumor", "tumor cells", "cancer" and "cancer cells", (used interchangeably) refer to cells which exhibit relatively autonomous growth, so that they exhibit an aberrant growth phenotype characterized by a significant loss of control of cell proliferation (i.e., de-regulated cell division). Neoplastic cells can be malignant or benign.

"Suppressing" tumor growth indicates a growth state that is curtailed when compared to growth without treatment or prevention as described herein. Tumor cell growth can be assessed by any means known in the art, including, but not limited to, measuring tumor size, determining whether tumor cells are proliferating using a ³H-thymidine incorporation assay, or counting tumor cells. "Suppressing" tumor cell growth means any or all of the following states: slowing, delaying, and stopping tumor growth, as well as tumor shrinkage.

"Host cell" or "recipient cell" is intended to include any individual cell or cell culture which can be or have been recipients for vectors or the incorporation of exogenous nucleic acid molecules, polynucleotides and/or peptides (or

polypeptides). It also is intended to include progeny of a single cell, and the progeny may not necessarily be completely identical (in morphology or in genomic or total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation. The cells may be procaryotic or eucaryotic, and include but are not limited to bacterial cells, yeast cells, animal cells, and mammalian cells, e.g., murine, rat, simian or human.

The term "isolated" means separated from constituents, cellular and otherwise, in which the polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, are normally associated with in nature. For example, with respect to a polynucleotide, an isolated polynucleotide is one that is separated from the 5' and 3' sequences with which it is normally associated in the chromosome. As is apparent to those of skill in the art, a non-naturally occurring polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, does not require "isolation" to distinguish it from its naturally occurring counterpart.

In addition, a "concentrated" "separated" or "diluted" polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, is distinguishable from its naturally occurring counterpart in that the concentration or number of molecules per volume is greater than "concentrated" or less than "separated" than that of its naturally occurring counterpart. A polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, which differs from the naturally occurring counterpart in its primary sequence or for example, by its glycosylation pattern, need not be present in its isolated form since it is distinguishable from its naturally occurring counterpart by its primary sequence, or alternatively, by another characteristic such as glycosylation pattern. Although not explicitly stated for each of the inventions disclosed herein, it is to be understood that all of the above embodiments for each of the compositions disclosed below and under the appropriate conditions, are provided by this invention. Thus, a non-naturally occurring polynucleotide is provided as a separate embodiment from the isolated naturally occurring polynucleotide. A protein produced in a bacterial cell is provided as a separate embodiment from the naturally occurring protein isolated from a eukaryotic cell in which it is produced in nature.

As used herein, the term "comprising" is intended to mean that the compositions and methods include the recited elements, but not excluding others. "Consisting essentially of" when used to define compositions and methods, shall mean excluding other elements of any essential significance to the combination.

5 Thus, a composition consisting essentially of the elements as defined herein would not exclude trace contaminants from the isolation and purification method and pharmaceutically acceptable carriers, such as phosphate buffered saline, preservatives, and the like. "Consisting of" shall mean excluding more than trace elements of other ingredients and substantial method steps for administering the compositions of this invention. Embodiments defined by each of these transition
10 terms are within the scope of this invention.

This invention provides improved cancer vaccines and methods of using the vaccines to induce an immune response to a native self-antigen in a subject. As shown in the experimental examples below, the compositions and methods of
15 this invention provide protective immunity against growth of tumor cells *in vivo* and a means to inhibit the growth of tumors *in vivo*. The methods also induce tumor reduction of established tumors *in vivo*. For purposes of immunization, heterologous/altered antigens can be delivered to antigen-presenting cells as protein/peptide or in the form of polynucleotide encoding the protein/peptide.
20 Antigen-presenting cells (APCs), as defined above, include but are not limited to dendritic cells (DCS), monocytes/macrophages, B-lymphocytes or other cell type(s) expressing the necessary MHC/co-stimulatory molecules. The methods described below focus primarily on DCS which are the most potent, preferred APCs.

25 This invention also provides an isolated novel heterologous or altered antigen that is capable of inducing an immune response against a self-antigen in a subject, an isolated nucleic acid encoding the antigen, as well as vectors and host cells containing the nucleic acids. Methods of replicating and expressing the isolated nucleic acids also are within the scope of this invention. Vectors and
30 methods for *in vitro* and *in vivo* transduction are briefly described below and are well known in the art. The incorporation and expression of the exogenous nucleic

acid can be confirmed using RT-PCR, Northern and Southern blotting analysis.

Sambrook et al. (1989) *Supra*.

The methods of the invention are exemplified below. Melanoma-associated antigens (MAAs) were used to transduce murine DCs which were then tested for their ability to activate cytotoxic T lymphocytes (CTLs) and induce protective immunity against B16 melanoma tumor cells. Dendritic cells derived from bone marrow displayed surface markers characteristic of DCs and were functionally active *in vitro* as determined in a mixed lymphocyte reaction and as indicated by their ability to induce primary antigen-specific proliferation of syngeneic T lymphocytes. The DCs were efficiently transduced with adenovirus type 2 (Ad2) based vectors while remaining phenotypically and functionally intact. Immunization of C57BL/6 mice with DCs transduced with Ad vector encoding the non-self human gp100 melanoma antigen (Ad2/hugp100) elicited the development of gp100-specific CTLs capable of lysing syngeneic fibroblasts transduced with Ad2/hugp100 as well as B16 cells expressing endogenous murine gp100. The induction of gp100-specific CTLs was associated with long-term protection against lethal subcutaneous challenge with B16 cells.

Although this invention is exemplified using heterologous gp100 melanoma tumor antigen, any heterologous or altered antigen is useful in the methods described herein.

For example, polypeptides and the polynucleotides encoding antigens of this invention can be, in one embodiment, the heterologous counterpart or an altered antigen of previously characterized tumor-associated antigens such as MUC-1 (Henderson et al. (1996) *Cancer Res.* **56**:3763); MART-1 (Kawakami et al. (1994) *Proc. Natl. Acad. Sci.* **91**:3515; Kawakami et al. (1997) *Intern. Rev. Immunol.* **14**:173; Ribas et al. (1997) *Cancer Res.* **57**:2865); HER-2/neu (U.S. Patent No. 5,550,214); MAGE (PCT/US92/04354); HPV16, 18E6 and E7 (Ressing et al. (1996) *Cancer Res.* **56**(1):582; Restifo (1996) *Current Opinion in Immunol.* **8**:658; Stern (1996) *Adv. Cancer Res.* **69**:175; Tindle et al. (1995) *Clin. Exp. Immunol.* **101**:265; van Driel et al. (1996) *Annals of Medicine* **28**:471); CEA (U.S. Patent No. 5,274,087); PSA (Lundwall, A. (1989) *Biochem. Biophys.*

Research Communications **161**:1151); prostate membrane specific antigen (PSMA) (Israeli et al. (1993) Cancer Research **53**:227); tyrosinase (U.S. Patent Nos. 5,530,096 and 4,898,814; Brichard et al. (1993) J. Exp. Med. **178**:489); tyrosinase related proteins 1 or 2 (TRP-1 and TRP-2); NY-ESO-1 (Chen et al. (1997) Proc. Natl. Acad. Sci. U.S.A. **94**:1914), or the GA733 antigen (U.S. Patent No. 5,185,254).

Also within the scope of this invention is an heterologous or altered antigen corresponding to an epitope or wild-type antigenic peptide corresponding to a yet unidentified protein. A common strategy in the search for tumor antigens is to isolate tumor-specific T-cells and attempt to identify the antigens recognized by these cells. In patients with cancer, specific CTLs have been derived from lymphocytic infiltrates present at the tumor site. Weidmann et al., *supra*. These TILs are unique cell population that can be traced back to sites of disease when they are labeled with indium and adoptively transferred. Alternatively, large libraries of putative antigens can be produced and tested. Using the "phage method" (Scott and Smith (1990) Science **249**:386; Cwirla et al. (1990) Proc. Natl. Acad. Sci. **87**:6387; and Devlin et al. (1990) Science **249**:404), very large libraries can be constructed. Another approach uses primarily chemical methods, of which the Geysen method (Geysen et al. (1986) Mol. Immunol. **23**:709; and Geysen et al. (1987) J. Immunol. Method **102**:259) and the method of Fodor et al. (1991) Science **251**:767, are examples. Furka et al. (1988) 14th Inter. Cong. Bio. Vol. **5**, Abst. FR:013; Furka (1991) Inter. J. Peptide Protein Res. **37**:487), Houghton (U.S. Patent No. 4,683,211, issued December 1986) and Rutter, et al. (U.S. Patent No. 5,010,175, issued April 23, 1991) describe methods to produce a mixture of peptides.

In a further aspect of this invention, Solid-**PH**ase Epitope **RE**covery ("SPHERE", described in PCT WO 97/35035) described below, can be used to identify tumor antigens and altered antigens corresponding to self antigens.

After identification and cloning of an altered antigen, the antigen or epitope can be expressed and purified for presentation to APC using the methods disclosed herein. In a further embodiment, the full-length native antigen can be

selectively modified to encode or present the altered epitope using methods known in the art, e.g. PCR directed mutagenesis. Sambrook et al., *supra*.

5 This invention further provides methods to elicit CD4⁺ and CD8⁺ T cells responses in a subject. The induction of this immune response also is a means to assay a positive response to the therapy. The presence of a large number of T-cells in tumor has been correlated with a prognostically favorable outcome in some cases (Whiteside and Parmiani (1994) *Cancer Immunol. Immunother.* 39:15). Woolley et al. (1995) *Immunology* 84:55, has shown that implantation of polyurethane sponges containing irradiated tumor cells can efficiently trap anti-tumor CTLs (4-times greater than lymph fluid, 50-times greater than spleen or peripheral blood). Following activation with T-cell cytokines in the presence of their appropriately presented recognition antigen, TILs proliferate in culture and acquire potent anti-tumor cytolytic properties. Weidmann et al. (1994) *Cancer Immunol. Immunother.* 39:1. Assays to determine T cell response are well known
10 in the art and any method that will compare T cell number and activity prior to and subsequent to therapy can be utilized. In addition, the induction of co-stimulatory cytokines by the heterologous/altered antigen could also stimulate pre-existing anergic or low affinity self-reactive CTL clones.

When the method is practiced *in vitro* as a screen to identify antigenic
20 peptides and nucleic acids of the invention, induction of cytotoxic T lymphocytes capable of lysing host tumor cells indicates that the antigenic peptide and/or nucleic acid of the screen is a potential therapeutic agent.

The methods of this invention can be further modified by co-administering more than one heterologous/altered antigen and/or an effective amount of a
25 cytokine or co-stimulatory molecule or other transgene to the subject.

The antigen is administered to the subject either as a nucleic acid coding for the peptide/protein or by administering APC presenting the antigen. In one embodiment, the APC is a dendritic cell which includes, but is not limited to a pulsed or genetically modified dendritic cell. When the method is practiced *in vitro*, the APC may be a foster antigen presenting cell. Methods of presenting the
30 antigen to the APC are described herein.

The APC can be further genetically modified to co-express a cytokine alone, or in combination with a co-stimulatory molecule or other transgene.

The APC expressing a heterologous and/or altered antigen also can be used to expand and isolate a population of immune effectors which, in turn, are useful for adoptive immunotherapy alone or as an adjuvant to the methods described above. As above, cytokines and/or co-stimulatory molecules or nucleic acids encoding them, can be co-administered with the immune effector cells. Alternatively, the immune effector cells can be genetically modified to express a foreign nucleic acid encoding a cytokine or co-stimulatory molecule. Prior to administration *in vivo*, the immune effector cells are screened *in vitro* for their ability to lyse tumor cells.

Furthermore, the invention provides a method for cloning the cDNA and genomic DNA encoding such protein by generating degenerate oligonucleotides probes or primers based on the sequence of the epitope. Compositions comprising the nucleic acid and a carrier, such as a pharmaceutically acceptable carrier, a solid support or a detectable label, are further provided by this method as well as methods for detecting the sequences in a sample using methods such as Northern analysis, Southern analysis and PCR.

Further provided by this invention are therapeutic and diagnostic comprising oligopeptide sequences determined according to the foregoing methods. Compositions comprising the oligopeptide sequence and a carrier, such as a pharmaceutically acceptable carrier, a solid support or a detectable label, are further provided by this method as well as methods for detecting the oligopeptide sequence in a sample using methods such as Western analysis and ELISA.

Harlow and Lane (1989) *supra*.

Materials and Methods

Identification of Tumor Associated Antigens

Any conventional method, e.g., subtractive library, comparative Northern and/or Western blot analysis of normal and tumor cells, Expression Cloning, Serial Analysis Gene Expression "SAGE" (U.S. Patent No. 5,695,937) and Solid

PHase Epitope REcovery "SPHERE" (described in PCT WO 97/35035), can be used to identify putative antigens for use in the subject invention.

SAGE analysis can be employed to identify the antigens recognized by expanded immune effector cells such as CTLs. SAGE analysis involves identifying nucleotide sequences expressed in the antigen-expressing cells. Briefly, SAGE analysis begins with providing complementary deoxyribonucleic acid (cDNA) from (1) the antigen-expressing population and (2) cells not expressing that antigen. Both cDNAs can be linked to primer sites. Sequence tags are then created, for example, using the appropriate primers to amplify the DNA. By measuring the differences in these tags between the two cell types, sequences which are over expressed in the antigen-expressing cell population can be identified.

Expression cloning methodology as described in Kawakami et al. (1994) PNAS 91:3515, also can be used to identify a novel tumor-associated antigen. Briefly, in this method, a library of cDNAs corresponding to mRNAs derived from tumor cells is cloned into an expression vector and introduced into target cells which are subsequently incubated with cytotoxic T cells. One identifies pools of cDNAs that are able to stimulate the CTL and through a process of sequential dilution and re-testing of less complex pools of cDNAs one is able to derive unique cDNA sequences that are able to stimulate the CTL and thus encode the cognate tumor antigen.

An antigen identification method, **SPHERE**, is described in PCT WO 97/35035. Briefly, an empirical screening method for the identification of MHC Class I-restricted CTL epitopes is described that utilizes peptide libraries synthesized on a solid support (*e.g.*, plastic beads) where each bead contains approximately 200 picomoles of a unique peptide that can be released in a controlled manner. The synthetic peptide library is tailored to a particular HLA restriction by fixing anchor residues that confer high-affinity binding to a particular HLA allele (*e.g.*, HLA-A2) but contain a variable TCR epitope repertoire by randomizing the remaining positions. Roughly speaking, 50 96-well plates with 10,000 beads per well will accommodate a library with a complexity

of approximately 5×10^7 . In order to minimize both the number of CTL cells required per screen and the amount of manual manipulations, the eluted peptides can be further pooled to yield wells with any desired complexity. Based on experiments with soluble libraries, it should be possible to screen 10^7 peptides in 96-well plates (10,000 peptides per well) with as few as 2×10^6 CTL cells. After cleaving a percentage of the peptides from the beads and incubating them with ^{51}Cr -labeled APCs (e.g., T2 cells) and the CTL line(s), peptide pools containing reactive species can be determined by measuring ^{51}Cr -release according to standard methods known in the art. Alternatively, cytokine production (e.g., interferon- γ) or proliferation (e.g., incorporation of ^3H -thymidine) assays may be used. After identifying reactive 10,000-peptide mixtures, the beads corresponding to those mixtures are separated into smaller pools and distributed to new 96-well plates (e.g., 100 beads per well). An additional percentage of peptide is released from each pool and re-assayed for activity by one of the methods listed above. Upon identification of reactive 100-peptide pools, the beads corresponding to those peptide mixtures are redistributed at 1 bead per well of a new 96-well plate. Once again, an additional percentage of peptide is released and assayed for reactivity in order to isolate the single beads containing the reactive library peptides. The sequence of the peptides on individual beads can be determined by sequencing residual peptide bound to the beads by, for example, N-terminal Edman degradation or other analytical techniques known to those of skill in the art.

In vitro confirmation of the immunogenicity of a putative antigen of this invention can be confirmed using the method described below which assays for the induction of CTLs.

After isolation of the epitope or antigen, it can be expressed and purified using methods known in the art.

Alternatively, muteins of the antigen as well as allogeneic and antigens from a different species, of previously characterized antigens are useful in the subject invention. For example, MART1 and gp100 are melanocyte differentiation antigens specifically recognized by HLA-A2 restricted tumor-

infiltrating lymphocytes (TILs) derived from patients with melanoma, and appear to be involved in tumor regression (Kawakami Y. et al. (1994) PNAS USA 91:6458 and Kawakami Y. et al. (1994) PNAS USA 91:91:3515). Recently, the mouse homologue of human MART-1 has been isolated. The full-length open reading frame of the mouse MART1 consists of 342 bp, encoding a protein of 113 amino acid residues with a predicted molecular weight of ~13 kDa. Alignment of human and murine MART1 amino acid sequences showed 68.6% identity.

The murine homologue of gp100 has also been identified. The open reading frame consists of 1,878 bp, predicting a protein of 626 amino acid residues which exhibits 75.5% identity to human gp100.

Additional antigens include, but are not limited to HER-2/neu (U.S. Patent No. 5,550,214); MAGE (PCT/US92/04354); HPV16, 18E6 and E7 (Ressing et al. (1996) Cancer Res. 56(1):582; Restifo (1996) Current Opinion in Immunol. 8:658; Stern (1996) Adv. Cancer Res. 69:175; Tindle et al. (1995) Clin. Exp. Immunol. 101:265; and van Driel et al. (1996) Annals of Medicine 28:471); CEA (U.S. Patent No. 5,274,087); PSA (Lundwall A. (1989) Biochem. Biophys. Research Communications 161:1151); prostate membrane specific antigen (PSMA) (Israeli et al. (1993) Cancer Research 53:227); tyrosinase (U.S. Patent Nos. 5,530,096 and 4,898,814, and Brichard et al. (1993) J. Exp. Med. 178:489); tyrosinase related proteins 1 or 2 (TRP-1 and TRP-2); NY-ESO-1 (Chen et al. (1997) PNAS 94:1914); or the GA733 antigen (U.S. Patent No. 5,185,254).

In vitro confirmation of the immunogenicity of a putative antigen of this invention can be confirmed using the method described below which assays for the generation of CTLs.

Isolation, Culturing and Expansion of APCs, Including Dendritic Cells

Various methods to isolate and characterize APCs including DCs have been known in the art. At least two methods have been used for the generation of human dendritic cells from hematopoietic precursor cells in peripheral blood or bone marrow. One approach utilizes the rare CD34+ precursor cells and stimulate them with GM-CSF plus TNF- α . The other method makes use of the more

abundant CD34- precursor population, such as adherent peripheral blood monocytes, and stimulate them with GM-CSF plus IL-4 (see, for example, Sallusto et al. (1994), *supra*).

5 In one aspect of the invention, the method described in Romani et al (1996), *supra*; and Bender et al (1996), *supra* is used to generate both immature and mature dendritic cells from the peripheral blood mononuclear cells (PBMC) of a mammal, such as a murine, simian or human. Briefly, isolated PBMC are pre-treated to deplete T- and B-cells by means of an immunomagnetic technique. Lymphocyte-depleted PBMC are then cultured for 7 days in RPMI medium,
10 supplemented with 1% autologous human plasma and GM-CSF/IL-4, to generate dendritic cells. Dendritic cells are nonadherence as opposed to their monocyte progenitor. Thus, on day 7, non-adherent cells are harvested for further processing.

The dendritic cells derived from PBMC in the presence of GM-CSF and
15 IL-4 are immature, in that they can lost the nonadherence property and revert back to macrophage cell fate if the cytokine stimuli are removed from the culture. The dendritic cells in an immature state are very effective in processing native protein antigens for the MHC class II restricted pathway (Romani et al. (1989) *J. Exp. Med.* 169:1169.

20 Further maturation of cultured dendritic cells is accomplished by culturing for 3 days in a macrophage-conditioned medium (CM), which contains the necessary maturation factors. Mature dendritic cells are less able to capture new proteins for presentation but are much better at stimulating resting T cells (both CD4+ and CD8+) to grow and differentiate.

25 Mature dendritic cells can be identified by their change in morphology, such as the formation of more motile cytoplasmic processes; by their nonadherence; by the presence of at least one of the following markers: CD83, CD68, HLA-DR or CD86; or by the loss of Fc receptors such as CD115 (reviewed in Steinman (1991) *Annu. Rev. Immunol.* 9:271.)

30 More specifically, the method requires collecting an enriched collection of white cells and platelets from leukapheresis that is then further fractionated by

countercurrent centrifugal elutriation (CCE) (Abrahamsen, T.G. et al. (1991) J. Clin. Apheresis. 6:48-53). Cell samples are placed in a special elutriation rotor. The rotor is then spun at a constant speed of, for example, 3000 rpm. Once the rotor has reached the desired speed, pressurized air is used to control the flow rate of cells. Cells in the elutriator are subjected to simultaneous centrifugation and a washout stream of buffer that is constantly increasing in flow rate. This results in fractional cell separations based largely but not exclusively on differences in cell size.

Quality control of APC and more specifically DC collection and confirmation of their successful activation in culture is dependent upon a simultaneous multi-color FACS analysis technique which monitors both monocytes and the dendritic cell subpopulation as well as possible contaminant T lymphocytes. It is based upon the fact that DCs do not express the following markers: CD3 (T cell); CD14 (monocyte); CD16, 56, 57 (NK/LAK cells); CD19, 20 (B cells). At the same time, DCs do express large quantities of HLA-DR, significant HLA-DQ and B7.2 (but little or no B7.1) at the time they are circulating in the blood (in addition they express Leu M7 and M9, myeloid markers which are also expressed by monocytes and neutrophils).

When combined with a third color reagent for analysis of dead cells, propidium iodide (PI), it is possible to make positive identification of all cell subpopulations (see Table 1):

TABLE 1
FACS analysis of fresh peripheral cell subpopulations

	<u>Color #1</u>	<u>Color #2</u>	<u>Color #3</u>
	<u>Cocktail</u>	<u>HLA-DR</u>	<u>PI</u>
	<u>3/14/16/19/20/56/57</u>		
Live Dendritic cells	Negative	Positive	Negative
Live Monocytes	Positive	Positive	Negative
Live Neutrophils	Negative	Negative	Negative
Dead Cells	Variable	Variable	Positive

Additional markers can be substituted for additional analysis:

Color #1: CD3 alone, CD14 alone, etc.; Leu M7 or Leu M9; anti-Class I, etc.

Color #2: HLA-Dq, B7.1, B7.2, CD25 (IL2r), ICAM, LFA-3, etc.

5 The goal of FACS analysis at the time of collection is to confirm that the DCs are enriched in the expected fractions, to monitor neutrophil contamination, and to make sure that appropriate markers are expressed. This rapid bulk collection of enriched DCs from human peripheral blood, suitable for clinical applications, is absolutely dependent on the analytic FACS technique described above for quality control. If need be, mature DCs can be immediately separated from monocytes at this point by fluorescent sorting for "cocktail negative" cells.

10 It may not be necessary to routinely separate DCs from monocytes because, as will be detailed below, the monocytes themselves are still capable of differentiating into DCs or functional DC-like cells in culture.

Once collected, the DC rich/monocyte APC fractions (usually 150 through 190) can be pooled and cryopreserved for future use, or immediately placed in

15 short term culture.

Alternatively, others have reported that a method for upregulating (activating) dendritic cells and converting monocytes to an activated dendritic cell phenotype. This method involves the addition of calcium ionophore to the culture media convert monocytes into activated dendritic cells. Adding the calcium

20 ionophore A23187, for example, at the beginning of a 24-48 hour culture period resulted in uniform activation and dendritic cell phenotypic conversion of the pooled "monocyte plus DC" fractions: characteristically, the activated population becomes uniformly CD14 (Leu M3) negative, and upregulates HLA-DR, HLA-DQ, ICAM-1, B7.1, and B7.2. Furthermore this activated bulk population

25 functions as well on a small numbers basis as a further purified.

Specific combination(s) of cytokines have been used successfully to amplify (or partially substitute) for the activation/conversion achieved with calcium ionophore: these cytokines include but are not limited to G-CSF, GM-CSF, IL-2, and IL-4. Each cytokine when given alone is inadequate for optimal

30 upregulation.

In one embodiment, the APCs and cells expressing one or more antigens are autologous. In another embodiment, the APCs and cells expressing the antigen are allogeneic. *i.e.*, derived from a different subject.

5 **Presentation of Antigen by the APC**

Peptide fragments from antigens must first be bound to peptide binding receptors (major histocompatibility complex class I and class II molecules) that display the antigenic peptides on the surface of the APCs. Palmer E. and Cresswell (1998) *Annu. Rev. Immunol.* **16**:323 and Germain R.N. (1996) *Immunol. Rev.* **151**:5. T lymphocytes produce an antigen receptor that they use to monitor the surface of APCs for the presence of foreign peptides. The antigen receptors on CD4⁺ T cells recognize antigenic peptides bound to MHC class II molecules whereas the receptors on CD8⁺ T cells react with antigens displayed on class I molecules. For a general review of the methods for presentation of exogenous antigen by APC, see Raychaudhuri and Rock (1998) *Nature Biotechnology* **16**:1025.

For purposes of immunization, antigens can be delivered to antigen-presenting cells as protein/peptide or in the form of polynucleotides encoding the protein/peptide *ex vivo* or *in vivo*. The methods described below focus primarily on DCs which are the most potent, preferred APCs.

Several different techniques have been described to produce genetically modified APCs. These include: (1) the introduction into the APCs of polynucleotides that express antigen or fragments thereof; (2) infection of APCs with recombinant vectors to induce endogenous expression of antigen; and (3) introduction of tumor antigen into the DC cytosol using liposomes. (See, Boczkowski D. et al. (1996) *J. Exp. Med.* **184**:465; Rouse et al. (1994) *J. Virol.* **68**:5685; and Nair et al. (1992) *J. Exp. Med.* **175**:609). For the purpose of this invention, any method which allows for the introduction and expression of the heterologous or non-self antigen and presentation by the MHC on the surface of the APC is within the scope of this invention.

Several techniques have been described for the presentation of exogenous protein and/or peptide by the APC. These techniques are briefly described below.

Antigen Pulsing

5 Pulsing is accomplished *in vitro/ex vivo* by exposing APCs to antigenic protein or peptide(s). The protein or peptide(s) are added to APCs at a concentration of 1-10 μ m for approximately 3 hours. Paglia et al. (1996) J. Exp. Med. **183**:317, has shown that APC incubated with whole protein *in vitro* were recognized by MHC class I-restricted CTLs, and that immunization of animals
10 with these APCs led to the development of antigen-specific CTLs *in vivo*.

Protein/peptide antigen can also be delivered to APC *in vivo* and presented by the APC. Antigen is preferably delivered with adjuvant via the intravenous, subcutaneous, intranasal, intramuscular or intraperitoneal route of delivery. Grant E.P. and Rock K.L. (1992) J. Immunol. **148**:13; Norbury, C. C. et al. (1995) Immunity **3**:783; and Reise-Sousa C. and Germain R.N. (1995) J. Exp. Med. **182**:841.
15

Antigen Painting

Another method which can be used is termed "painting". It has been demonstrated that glycosyl-phosphatidylinositol (GPI)-modified proteins possess the ability to reincorporate themselves back into cell membranes after purification. Hirose et al. (1995) *Methods Enzymol.* **250**:582; Medof et al. (1984) *J. Exp. Med.* **160**:1558; Medof (1996) *FASEB J.* **10**:574; and Huang et al. (1994) *Immunity* **1**:607, have exploited this property in order to create APCs of specific composition for the presentation of antigen to CTLs. Expression vectors for β 2-microglobulin and the HLA-A2.1 allele were first devised. The proteins were expressed in Schneider S2 *Drosophila melanogaster* cells, known to support GPI-modification. After purification, the proteins could be incubated together with a purified antigenic peptide which resulted in a trimolecular complex capable of efficiently inserting itself into the membranes of autologous cells. In essence, these protein mixtures were used to "paint" the APC surface, conferring the ability to stimulate a CTL clone that was specific for the antigenic peptide. Cell coating was shown to occur rapidly and to be protein concentration dependent. This method of generating APCs bypasses the need for gene transfer into the APC and permits control of antigenic peptide densities at the cell surfaces.

Foster Antigen Presenting Cells

Foster APCs are derived from the human cell line 174xCEM.T2, referred to as T2, which contains a mutation in its antigen processing pathway that restricts the association of endogenous peptides with cell surface MHC class I molecules (Zweerink et al. (1993) *J. Immunol.* **150**:1763). This is due to a large homozygous deletion in the MHC class II region encompassing the genes TAP1, TAP2, LMP1, and LMP2, which are required for antigen presentation to MHC class I-restricted CD8⁺ CTLs. In effect, only "empty" MHC class I molecules are presented on the surface of these cells. Exogenous peptide added to the culture medium binds to these MHC molecules provided that the peptide contains the allele-specific binding motif. These T2 cells are referred to herein as "foster"

APCs. They can be used in conjunction with this invention to present the heterologous, altered or control antigen.

Transduction of T2 cells with specific recombinant MHC alleles allows for redirection of the MHC restriction profile. Libraries tailored to the recombinant allele will be preferentially presented by them because the anchor residues will prevent efficient binding to the endogenous allele.

High level expression of MHC molecules makes the APC more visible to the CTLs. Expressing the MHC allele of interest in T2 cells using a powerful transcriptional promoter (e.g., the CMV promoter) results in a more reactive APC (most likely due to a higher concentration of reactive MHC-peptide complexes on the cell surface).

Expansion of Immune Effector Cells

In one embodiment, the present invention makes use of these APCs to stimulate production of an enriched population of antigen-specific immune effector cells. The antigen-specific immune effector cells are expanded at the expense of the APCs, which die in the culture. The process by which naïve immune effector cells become educated by other cells is described essentially in Coulie (1997) *Molec. Med. Today* 3: 261. The substantially pure population of educated, antigen-specific immune effector cells produced by this method are useful to cause tumor regression.

The APCs (e.g., DCs) presenting the heterologous/altered antigen are mixed with naïve immune effector cells. Preferably, the cells may be cultured in the presence of a cytokine, for example IL2. Because dendritic cells secrete potent immunostimulatory cytokines, such as IL-12, it may not be necessary to add supplemental cytokines during the first and successive rounds of expansion. In any event, the culture conditions are such that the antigen-specific immune effector cells expand (*i.e.*, proliferate) at a much higher rate than the APCs. Multiple infusions of APCs and optional cytokines can be performed to further expand the population of antigen-specific cells.

In one embodiment, the immune effector cells are T cells. In a separate embodiment, the immune effector cells can be genetically modified by transduction with a transgene coding for example, IL-2, IL-11 or IL-13. Methods for introducing transgenes *in vitro*, *ex vivo* and *in vivo* are well known in the art. See Sambrook et al. (1989) *supra*.

An effector cell population suitable for use in the methods of the present invention can be autologous or allogeneic, preferably autologous. When effector cells are allogeneic, preferably the cells are depleted of alloreactive cells before use. This can be accomplished by any known means, including, for example, by mixing the allogeneic effector cells and a recipient cell population and incubating them for a suitable time, then depleting CD69⁺ cells, or inactivating alloreactive cells, or inducing anergy in the alloreactive cell population.

Hybrid immune effector cells can also be used. Immune effector cell hybrids are known in the art and have been described in various publications. See, for example, International Patent Application Nos. WO 98/46785 and WO 95/16775.

The effector cell population can comprise unseparated cells, i.e., a mixed population, for example, a PBMC population, whole blood, and the like. The effector cell population can be manipulated by positive selection based on expression of cell surface markers, negative selection based on expression of cell surface markers, stimulation with one or more antigens *in vitro* or *in vivo*, treatment with one or more biological modifiers *in vitro* or *in vivo*, subtractive stimulation with one or more antigens or biological modifiers, or a combination of any or all of these.

Effector cells can be obtained from a variety of sources, including but not limited to, PBMC, whole blood or fractions thereof containing mixed populations, spleen cells, bone marrow cells, tumor infiltrating lymphocytes, cells obtained by leukapheresis, biopsy tissue, lymph nodes, e.g., lymph nodes draining from a tumor. Suitable donors include an immunized donor, a non-immunized (naïve) donor, treated or untreated donors. A "treated" donor is one that has been

exposed to one or more biological modifiers. An "untreated" donor has not been exposed to one or more biological modifiers.

Methods of extracting and culturing effector cells are well known. For example, effector cells can be obtained by leukapheresis, mechanical apheresis using a continuous flow cell separator. For example, lymphocytes and monocytes can be isolated from the buffy coat by any known method, including, but not limited to, separation over Ficoll-Hypaque™ gradient, separation over a Percoll gradient, or elutriation. The concentration of Ficoll-Hypaque™ can be adjusted to obtain the desired population, for example, a population enriched in T cells.

Other methods based on affinity are known and can be used. These include, for example, fluorescence-activated cell sorting (FACS), cell adhesion, magnetic bead separation, and the like. Affinity-based methods may utilize antibodies, or portions thereof, which are specific for cell-surface markers and which are available from a variety of commercial sources, including, the American Type Culture Collection (Manassas, VA). Affinity-based methods can alternatively utilize ligands or ligand analogs, of cell surface receptors.

The effector cell population can be subjected to one or more separation protocols based on the expression of cell surface markers. For example, the cells can be subjected to positive selection on the basis of expression of one or more cell surface polypeptides, including, but not limited to, "cluster of differentiation" cell surface markers such as CD2, CD3, CD4, CD8, TCR, CD45, CD45RO, CD45RA, CD11b, CD26, CD27, CD28, CD29, CD30, CD31, CD40L; other markers associated with lymphocyte activation, such as the lymphocyte activation gene 3 product (LAG3), signaling lymphocyte activation molecule (SLAM), T1/ST2; chemokine receptors such as CCR3, CCR4, CXCR3, CCR5; homing receptors such as CD62L, CD44, CLA, CD146, $\alpha 4\beta 7$, $\alpha E\beta 7$; activation markers such as CD25, CD69 and OX40; and lipoglycans presented by CD1. The effector cell population can be subjected to negative selection for depletion of non-T cells and/or particular T cell subsets. Negative selection can be performed on the basis of cell surface expression of a variety of molecules, including, but not limited to,

B cell markers such as CD19, and CD20; monocyte marker CD14; the NK cell marker CD56.

An effector cell population can be manipulated by exposure, *in vivo* or *in vitro*, to one or more biological modifiers. Suitable biological modifiers include, but are not limited to, cytokines such as IL-2, IL-4, IL-10, TNF- α , IL-12, IFN- γ ; non-specific modifiers such as phytohemagglutinin (PHA), phorbol esters such as phorbol myristate acetate (PMA), concanavalin-A, and ionomycin; antibodies specific for cell surface markers, such as anti-CD2, anti-CD3, anti-IL2 receptor, anti-CD28; chemokines, including, for example, lymphotactin. The biological modifiers can be native factors obtained from natural sources, factors produced by recombinant DNA technology, chemically synthesized polypeptides or other molecules, or any derivative having the functional activity of the native factor. If more than one biological modifier is used, the exposure can be simultaneous or sequential.

The present invention provides compositions comprising immune effector cells, which may be T cells, enriched in antigen-specific cells, specific for a peptide of the invention. By "enriched" is meant that a cell population is at least about 50-fold, more preferably at least about 500-fold, and even more preferably at least about 5000-fold or more, enriched from an original naive cell population. The proportion of the enriched cell population which comprises antigen-specific cells can vary substantially, from less than 10% up to 100% antigen-specific cells. If the cell population comprises at least 50%, preferably at least 70%, more preferably at least 80%, and even more preferably at least 90%, antigen-specific immune effector cells, specific for a peptide of the invention, then the population is said to be "substantially pure". The percentage which are antigen-specific can readily be determined, for example, by a ^3H -thymidine uptake assay or cytokine release assay in which the effector cell population (for example, a T-cell population) is challenged by an antigen-presenting matrix presenting an antigenic peptide of the invention.

Assaying Antigen-Specificity

An *in vitro* system will be needed to test or confirm which version of the modified or heterologous tumor antigen is most likely to be immunogenic in humans or the test subject. In this system, DCs will be used to present antigen to autologous peripheral blood lymphocytes. The DCs can be pulsed or transduced. Various culture conditions have been described that will support the generation of effector cells in cultures of DCs and lymphocytes. After several rounds of stimulation, the effector cells generated are tested for their ability to recognize native tumor antigen. Fewer rounds of stimulation may be required for antigens with high immunogenic potential. Both T helper (CD4⁺) and cytolytic effector cells (CD8⁺) can be elicited. The development of TAA-specific cells can be measured by several methods including proliferation or cytokine production (e.g. TNF- α , interferon- γ) upon exposure to TAA or lysis of TAA-expressing target cells as assessed by release of various intracellular labels/markers such as ⁵¹Chromium or lactose dehydrogenase (LDH). The antigen that induces the strongest response (in particular cytolytic activity) against the native human antigen would then be selected for immunization purposes.

In addition to previously identified and characterized antigens and epitopes, the methods of this invention also can use newly identified antigens which can be identified as exemplified below.

Production of Epitope or Antigen

Most preferably, heterologous/altered antigens and peptides of the present invention can be synthesized using an appropriate solid state synthetic procedure. Steward and Young, Solid Phase Peptide Synthesis, Freeman, San Francisco, Calif. (1968). A preferred method is the Merrifield process. Merrifield, Recent Progress in Hormone Res. 23:451 (1967). The antigenic activity of these peptides may conveniently be tested using, for example, the assays as described herein.

Once an isolated peptide of the invention is obtained, it may be purified by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any

other standard technique for protein purification. For immunoaffinity chromatography, an epitope may be isolated by binding it to an affinity column comprising antibodies that were raised against that peptide, or a related peptide of the invention, and were affixed to a stationary support.

5 Alternatively, affinity tags such as hexa-His (Invitrogen), Maltose binding domain (New England Biolabs), influenza coat sequence (Kolodziej et al. (1991) Methods Enzymol. **194**:508), and glutathione-S-transferase can be attached to the peptides of the invention to allow easy purification by passage over an appropriate affinity column. Isolated peptides can also be physically characterized using such
10 techniques as proteolysis, nuclear magnetic resonance, and x-ray crystallography.

 Also included within the scope of the invention are antigenic peptides that are differentially modified during or after translation, e.g., by phosphorylation, glycosylation, crosslinking, acylation, proteolytic cleavage, linkage to an antibody molecule, membrane molecule or other ligand, (Ferguson et al. (1988) Ann. Rev.
15 Biochem. **57**:285).

 Another aspect of the invention encompasses isolated nucleic acid sequences that encode the novel antigenic peptides described herein. With regard to nucleic acid sequences of the present invention, "isolated" means: an RNA or DNA polymer, portion of genomic nucleic acid, cDNA, or synthetic nucleic acid
20 which, by virtue of its origin or manipulation: (i) is not associated with all of a nucleic acid with which it is associated in nature (e.g. is present in a host cell as a portion of an expression vector); or (ii) is linked to a nucleic acid or other chemical moiety other than that to which it is linked in nature; or (iii) does not occur in nature. By "isolated" it is further meant a nucleic acid sequence: (i)
25 amplified *in vitro* by, for example, polymerase chain reaction (PCR); (ii) synthesized by, for example, chemical synthesis; (iii) recombinantly produced by cloning; or (iv) purified, as by cleavage and gel separation.

 The nucleic acid sequences of the present invention may be characterized, isolated, synthesized and purified using no more than ordinary skill. See
30 Sambrook et al. (1989) *supra*.

Compositions

This invention also provides compositions containing any of the above-mentioned proteins, muteins, polypeptides, nucleic acid molecules, vectors, cells, antibodies and fragments thereof, and an acceptable solid or liquid carrier. When the compositions are used pharmaceutically, they are combined with a "pharmaceutically acceptable carrier" for diagnostic and therapeutic use. These compositions also can be used for the preparation of medicaments for the diagnosis and treatment of diseases such as cancer.

Tumor Protection in Animal Models

The murine B16 melanoma model was used. In this model, C57BL/6 mice were immunized with bone marrow-derived DCs transduced with an Ad vector encoding either human gp100 (Ad/hugp100) or mouse gp100 (Ad/mgpl00). Mice immunized against heterologous human gp100 developed a protective immune response and were resistant to a lethal subcutaneous challenge of B16 melanoma cells (syngeneic tumor cell line that expresses gp100). In contrast, mice immunized with homologous mouse gp100 failed to mount a protective immune response against B16 melanoma cells and developed tumors at the site of B16 cell injection. This finding illustrates the difficulty in breaking tolerance against a self antigen (mouse gp100). The corresponding heterologous antigen from a different species (human gp100), however, is likely to contain several Class I and Class II-associated epitopes that will be recognized as foreign and elicit CD8⁺ and CD4⁺ T cell responses, respectively. The induction of cross-reactive CTLs that recognize both the heterologous and homologous self-antigen can then lead to lysis of host tumor cells.

Unfortunately, this type of animal model cannot be used to test the efficacy of modified or heterologous tumor antigens being considered for use in humans since mice and humans recognize different epitopes, primarily as a result of differences in their MHC molecules. It may be possible, however, to use the allogeneic human peripheral blood lymphocyte - severe combined immunodeficiency mouse (Hu-PBL-SCID) model. SCID mice lack mature B and

T lymphocytes and can be reconstituted with human PBLs. It may be possible to immunize such mice with test antigen to induce a response in adoptively transferred human PBLs and evaluate protection against challenge with a human tumor cell line (Mosier et al. (1988) *Nature* **335**:256; Parney et al. (1997) *Human Gene Therapy* **8**:1073; and Albert et al. (1997) *J. Immunol.* **159**:1393).

Another possibility is immunization of HLA-A2.1 transgenic mice to reproduce the immune reactivity of HLA-A2 individuals (Wentworth et al. (1996) *Eur. J. Immunol.* **26**:97).

Vectors Useful in Genetic Modifications

In general, genetic modifications of cells employed in the present invention are accomplished by introducing a vector containing a polypeptide or transgene encoding a heterologous or an altered antigen. A variety of different gene transfer vectors, including viral as well as non-viral systems can be used. Viral vectors useful in the genetic modifications of this invention include, but are not limited to adenovirus, adeno-associated virus vectors, retroviral vectors and adeno-retroviral chimeric vectors. APC and immune effector cells can be modified using the methods described below or by any other appropriate method known in the art.

Construction of Recombinant Adenoviral Vectors or Adeno-Associated Virus Vectors

Adenovirus and adeno-associated virus vectors useful in the genetic modifications of this invention may be produced according to methods already taught in the art. (see, e.g., Karlsson et al. (1986) *EMBO* **5**:2377; Carter (1992) *Current Opinion in Biotechnology* **3**:533; Muzyczka (1992) *Current Top. Microbiol. Immunol.* **158**:97; and *GENE TARGETING: A PRACTICAL APPROACH* (1992) ed. A. L. Joyner, Oxford University Press, NY). Several different approaches are feasible. Preferred is the helper-independent replication deficient human adenovirus system.

The recombinant adenoviral vectors based on the human adenovirus 5 (Virology 163:614, 1988) are missing essential early genes from the adenoviral genome (usually E1A/E1B). and are therefore unable to replicate unless grown in permissive cell lines that provide the missing gene products *in trans*. In place of the missing adenoviral genomic sequences, a transgene of interest can be cloned and expressed in cells infected with the replication deficient adenovirus.

Although adenovirus-based gene transfer does not result in integration of the transgene into the host genome (less than 0.1% adenovirus-mediated transfections result in transgene incorporation into host DNA), and therefore is not stable, adenoviral vectors can be propagated in high titer and transfect non-replicating cells. Human 293 cells, which are human embryonic kidney cells transformed with adenovirus E1A/E1B genes, typify useful permissive cell lines. However, other cell lines which allow replication-deficient adenoviral vectors to propagate therein can be used, including HeLa cells.

Additional references describing adenovirus vectors and other viral vectors which could be used in the methods of the present invention include the following: Horwitz, M.S., Adenoviridae and Their Replication, in Fields, B., et al. (eds.) VIROLOGY, Vol. 2, Raven Press New York, pp. 1679-1721, 1990); Graham F. et al., pp. 109-128 in METHODS IN MOLECULAR BIOLOGY, Vol. 7: GENE TRANSFER AND EXPRESSION PROTOCOLS, Murray E. (ed.) Humana Press, Clifton, N.J. (1991); Miller N. et al. (1995) FASEB Journal 9:190; Schreier H (1994) Pharmaceutica Acta Helvetiae 68:145; Schneider and French (1993) Circulation 88:1937; Curiel D.T. et al. (1992) Human Gene Therapy 3:147; Graham F.L. et al., WO 95/00655; Falck-Pedersen WO 95/16772; Deneffe P. et al. WO 95/23867; Haddada H. et al. WO 94/26914; Perricaudet M. et al. WO 95/02697; and Zhang W. et al. WO 95/25071. A variety of adenovirus plasmids are also available from commercial sources, including, e.g., Microbix Biosystems of Toronto, Ontario (see, e.g., Microbix Product Information Sheet: Plasmids for Adenovirus Vector Construction, 1996). See also, the papers by Vile et al. (1997) Nature Biotechnology 15:840; and Feng et al. (1997) Nature Biotechnology. 15:866, describing the construction and use of adeno-retroviral chimeric vectors that can be employed for genetic modifications.

Additional references describing AAV vectors which could be used in the methods of the present invention include the following: Kotin R. (1994) Human Gene Therapy 5:793; Flotte T.R. et al. (1995) Gene Therapy 2:357; Allen J.M. WO 96/17947; and Du et al. (1996) Gene Therapy 3:254.

5 APCs can be transduced with viral vectors encoding a relevant antigen. The most common viral vectors include recombinant poxviruses such as vaccinia and fowlpox virus (Bronte et al. (1997) PNAS 94:3183; and Kim et al. (1997) J. Immunother. 20:276) and, preferentially, adenovirus (Arthur et al. (1997) J. Immunol. 159:1393; Wan et al. (1997) Human Gene Therapy 8:1355; and Huang
10 et al. (1995) J. Virol. 69:2257). Retrovirus also may be used for transduction of human APCs (Marin et al. (1996) J. Virol. 70:2957).

In vitro/ex vivo, exposure of human DCs to adenovirus (Ad) vector at a multiplicity of infection (MOI) of 500 for 16-24 hours in a minimal volume of serum-free medium reliably gives rise to transgene expression in 90-100% of
15 DCs. The efficiency of transduction of DCs or other APCs can be assessed by immunofluorescence using fluorescent antibodies specific for the tumor antigen being expressed (Kim et al. (1997) J. Immunother. 20:276). Alternatively, the antibodies can be conjugated to an enzyme (e.g. HRP) giving rise to a colored product upon reaction with the substrate. The actual amount of antigen being
20 expressed by the APCs can be evaluated by ELISA.

Transduced APCs can subsequently be administered to the host via an intravenous, subcutaneous, intranasal, intramuscular or intraperitoneal route of delivery.

In vivo transduction of DCs, or other APCs, can be accomplished by
25 administration of Ad (or other viral vectors) via different routes including intravenous, intramuscular, intranasal, intraperitoneal or cutaneous delivery. The preferred method is cutaneous delivery of Ad vector at multiple sites using a total dose of approximately 1×10^{10} - 1×10^{12} i.u. Levels of *in vivo* transduction can be
roughly assessed by co-staining with antibodies directed against APC marker(s)
30 and the antigen being expressed. The staining procedure can be carried out on biopsy samples from the site of administration or on cells from draining lymph

nodes or other organs where APCs (in particular DCs) may have migrated (Condon et al. (1996) Nature Med. 2:1122; Wan et al. (1997) Human Gene Therapy 8:1355). The amount of antigen being expressed at the site of injection or in other organs where transduced APCs may have migrated can be evaluated by ELISA on tissue homogenates.

Although viral gene delivery is more efficient, DCs can also be transduced *in vitro/ex vivo* by non-viral gene delivery methods such as electroporation, calcium phosphate precipitation or cationic lipid/plasmid DNA complexes (Arthur et al. (1997) Cancer Gene Therapy 4:17). Transduced APCs can subsequently be administered to the host via an intravenous, subcutaneous, intranasal, intramuscular or intraperitoneal route of delivery.

In vivo transduction of DCs, or other APCs, can potentially be accomplished by administration of cationic lipid/plasmid DNA complexes delivered via the intravenous, intramuscular, intranasal, intraperitoneal or cutaneous route of administration. Gene gun delivery or injection of naked plasmid DNA into the skin also leads to transduction of DCs (Condon et al. (1996) Nature Med. 2:1122; and Raz et al. (1994) PNAS 91:9519). Intramuscular delivery of plasmid DNA may also be used for immunization (Rosato et al. (1997) Human Gene Therapy 8:1451).

The transduction efficiency and levels of transgene expression can be assessed as described herein.

Administration Methods

Dendritic cells derived from peripheral blood of a subject such as a human patient are transduced with adenovirus vector encoding the tumor antigen using a multiplicity of infection of 200-500. Approximately 24 hours after infection, the transfected dendritic cells (10×10^7 cells) are administered to the patient iv or subcutaneously. The process is repeated 3-4 weeks later with up to 6 administrations of dendritic cells. Since it is possible to freeze dendritic cells and administer thawed cells, the subject does not have to be leukapheresed each time.

The agents identified herein as effective for their intended purpose can be administered to subjects having tumors or to individuals susceptible to or at risk of developing a tumor by inducing an immune response against the tumor. When the agent is administered to a subject such as a mouse, a rat or a human patient, the agent can be added to a pharmaceutically acceptable carrier and systemically or topically administered to the subject. To determine patients that can be beneficially treated, a tumor regression can be assayed. Therapeutic amounts can be empirically determined and will vary with the pathology being treated, the subject being treated and the efficacy and toxicity of the therapy.

Administration *in vivo* can be effected in one dose, continuously or intermittently throughout the course of treatment. Methods of determining the most effective means and dosage of administration are well known to those of skill in the art and will vary with the composition used for therapy, the purpose of the therapy, the target cell being treated, and the subject being treated. Single or multiple administrations can be carried out with the dose level and pattern being selected by the treating physician. Suitable dosage formulations and methods of administering the agents can be found below.

The agents and compositions of the present invention can be used in the manufacture of medicaments and for the treatment of humans and other animals by administration in accordance with conventional procedures, such as an active ingredient in pharmaceutical compositions.

More particularly, an agent of the present invention also referred to herein as the active ingredient, may be administered for therapy by any suitable route including nasal, topical (including transdermal, aerosol, buccal and sublingual), parental (including subcutaneous, intramuscular, intravenous and intradermal) and pulmonary. It will also be appreciated that the preferred route will vary with the condition and age of the recipient, and the disease being treated.

Adoptive Immunotherapy and Vaccines

The expanded populations of antigen-specific immune effector cells of the present invention also find use in adoptive immunotherapy regimes and as

vaccines. Thus, tumors expressing the antigen can be eradicated using the methods and compositions described herein.

Adoptive immunotherapy methods involve, in one aspect, administering to a subject a substantially pure population of educated, antigen-specific immune effector cells made by culturing naïve immune effector cells with APCs as described above. Preferably, the APCs are dendritic cells. In one embodiment, the adoptive immunotherapy methods described herein are autologous. In this case, the APCs are made using cells isolated from a single subject. The expanded population also employs T cells isolated from that subject. Finally, the expanded population of antigen-specific cells is administered to the same patient.

In a further embodiment, APCs or immune effector cells are administered with an effective amount of a stimulatory cytokine, such as IL-2 or a co-stimulatory molecule.

The following examples are intended to illustrate, but not limit the invention.

Experimental Examples

Animals and cell lines

Female C57BL/6 mice were purchased from Taconic (Germantown, NY) and were used at 8-12 weeks of age. Syngeneic SV40-transformed fibroblasts (SVB6KHA) have been described elsewhere (Gooding L.R. (1979) J. Immunol. 122:1002) and were a gift from Dr. Linda Gooding (Emory University, Atlanta, GA). The B16.F10 melanoma cell line syngeneic to C57BL/6 mice was obtained from the National Cancer Institute (Bethesda, MD). For injection, B16.F10 cells (1.5×10^4 cells) were resuspended in phosphate-buffered saline (PBS) and delivered to the abdomen subcutaneously (s.c.) in a 100 μ l volume. Tumor size was measured with electronic digital calipers 3 times per week starting around day 10. Tumors $\geq 3 \text{ mm}^2$ in size were scored as positive.

Adenoviral vectors

All recombinant adenovirus (Ad) vectors used were derived from Ad serotype 2 from which the E1 region was deleted and replaced with an expression cassette containing a cytomegalovirus (CMV) promoter driving expression of the transgene. The vector encoding β -Galactosidase (Ad2/ β Gal-4) and human gp100 (Ad2/hugp100vl) contained intact E3 and E4 regions (Armentano D. (1997) J. Virol. **71**:2408 and Zhai Y. (1996) J. Immunol. **156**:7001. The vector encoding murine gp100 (Ad2/mgp100) or vector lacking a transgene (Ad2/empty vector), possessed an intact E3 region with an E4 region modified by removal of all open reading frames and replacement with the E4 open reading frame 6 and protein IX moved from its original location (Armentano D. (1985) Human Gene Therapy **6**:1343). Finally, the Ad vector encoding murine tyrosinase-related protein 2 (Ad2/mTRP-2) contained an intact E4 region but was deleted for E3. The E2 region was left intact in all vectors.

Adenoviral particles were gradient-purified as previously described (Armentano D. (1985) Human Gene Therapy **6**:1343) and titers were determined by end-point dilution on 293 cells using fluorescent isothiocyanate (FITC)-conjugated anti-hexon antibody (Rich D.P. (1993) Human Gene Therapy **4**:461).

Preparation of bone marrow-derived dendritic cells

Dendritic cells (DCs) were prepared from bone marrow essentially as described by Inaba et al. (Inaba K. (1992) J. Exp. Med. **176**:1693). Briefly, bone marrow was flushed from the tibias and femurs of C57BL/6 mice and depleted of erythrocyte with commercial lysis buffer (Sigma, St. Louis, MO). Bone marrow cells were then treated with a cocktail of antibodies (Pharmingen, San Diego, CA) directed against CD8 (clone 53-6.7), CD4 (clone GK1.5), CD45R/B220 (clone RA3-6B2), Ly-6G/Gr-1 (clone RB6-8C5) and Ia (clone KH74) followed by rabbit complement (Accurate Chemical and Scientific Corporation, Westbury, N.Y.) to deplete lymphocytes, granulocytes and Ia⁺ cells. The remaining cells were cultured for 6 days in 6-well plates in RPMI-1640 medium (Gibco, Grand Island,

NY) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 10% fetal calf serum (FCS) and 100 ng/ml recombinant mouse GM-CSF (Genzyme, Cambridge, MA). Loosely adherent DCs were then collected, replated in 100 mm dishes and cultured in the same medium for another 24 hours after removal of contaminating non-adherent cells. This final DC population was then collected for FACS analysis and transduction with Ad vector.

For analysis of surface markers, DCs were first incubated with unlabeled antibodies (Pharmingen) specific for the major histocompatibility complex (MHC) Class I (clone AF6-88.5) and Class II (clone AF6-120.1) molecules, the co-stimulatory molecules B7.1 (CD80; clone IG10) and B7.2 (CD86; clone GL-1), the adhesion molecule ICAM-1 (CD54; clone 3E2), the integrin CD 11 c (clone 3E2) and the myeloid surface marker CD13 (clone R3-242). The cells were then counterstained with FITC-conjugated antibodies specific for the primary antibody. FACS analysis of the stained cells was performed on an EPICS Profile Analyzer from Coulter.

Transduction of DCs with Ad vector was conducted in 6-well plates with 4×10^6 DCs/well in a 3 ml volume of RPMI-1640 medium containing 10% FCS and 100 ng/ml GM-CSF. Virus was added to the wells at a multiplicity of infection (MOI) of 500 and the DCs were collected after 18-24 hours of incubation. For injection, transduced DCs were washed and resuspended in a 100 µl volume of PBS and delivered either s.c. to the abdomen or intravenously (i.v.) into the tail vein as specified in the text.

Cytotoxic T cell assay

To evaluate levels of cytotoxic T lymphocyte (CTL) activity, spleen cells from mice in the same treatment group (3 mice/group) were pooled and stimulated *in vitro* with syngeneic SVB6KHA fibroblasts transduced with Ad2 vector at an MOI of 100 for 24 hours. Cells were cultured in 24-well plates containing 5×10^6 spleen cells and 0.8 - 1.5×10^5 stimulator fibroblasts per well in a 2 ml volume. Cytolytic activity was assayed after 6 days of incubation. Target cells consisted of B16 melanoma cells and fibroblasts untransduced or transduced with virus at

an MOI of 100 for 48 hours. Targets were treated with 100 U/ml recombinant mouse γ -interferon (Genzyme) for 24 hours labeled with ^{51}Cr (51-Cr; New England Nuclear) overnight ($30 \mu\text{Ci}/10^5$ cells) and plated in round bottom 96 well plates at 5×10^3 cells/well. Effector cells were added at various effector:target (E:T) cell ratios in triplicate. The total reaction volume was kept constant at 200 μl /well. After 5 hours of incubation of effector and target cells at $37^\circ\text{C}/5\% \text{CO}_2$, 25 μl of cell-free supernatant was collected from each well and counted in a MicroBeta Trilux Liquid Scintillation Counter (Wallac Inc., Gaithersburg, MD). The amount of ^{51}Cr spontaneously released was obtained by incubating target cells in medium alone and the total amount of ^{51}Cr incorporated was determined by adding 1% Triton X-100 in distilled water. The percentage lysis was calculated as follows:

$$\% \text{ Lysis} = \frac{(\text{Sample cpm}) - (\text{Spontaneous cpm})}{(\text{Total cpm}) - (\text{Spontaneous cpm})} \times 100$$

ELISPOT assay

The frequency of splenic T lymphocytes reactive with gp100 was evaluated in an ELISPOT assay. Spleen cells from mice immunized with Ad2/hugp100- or Ad2/empty vector-transduced DCs (4 mice/group) were pool and stimulated with H-2^b-restricted CTL epitopes derived from human gp100 (KVPRNQDWL), murine gp100 (EGSRNQDWL) or ovalbumin as a negative control (SIINFEKL). (For human and murine gp100 peptides, see Overwijk et al. (1998) J. Exp. Med. **188**: 277-286; for ovalbumin peptide, see Brossart et al. (1997) J. Immunol. **158**: 3270-3276.)

After 4 hours of stimulation with peptide, the spleen cells were transferred to 96-well nitrocellulose filter plates coated with γ -interferon-specific antibodies. After 40 hours of incubation, the cells were removed by washing and biotinylated antibodies against γ -interferon were added to the wells. The subsequent addition of streptavidin-alkaline phosphatase gave rise to dark spots corresponding to γ -interferon-producing cells.

Experimental Results

Characterization of bone-marrow derived dendritic cells and transduction by adenovirus vectors

Dendritic cells (DCs) derived from mouse bone marrow exhibited the veiled dendrite morphology typical of DCs and displayed a characteristic set of DC surface markers (Crowley M. (1989) Cell. Immunol. **118**:108) as determined by FACS analysis (Table 2). The cells expressed high levels of the major histocompatibility (MHC) Class I and Class II molecules, the co-stimulatory molecules B7.1 and B7.2, the ICAM-I adhesion molecule, the integrin CD11c and the CD13 myeloid surface marker. Transduction of DCs with recombinant Ad2-based vectors was achieved reproducibly with an efficiency of 90% or greater. Transduction did not affect the distribution of DC surface markers significantly except for a reproducible increase in levels of MHC Class I molecules (Table 2).

Table 2. FACS analysis of dendritic cell surface markers

DC sample	B7.1	B7.2	MHC I	MHC II	ICA MI	CD1 1c	CD13
Untransduced	80	77	41	70	96	81	80
Transduced	84	83	85	79	94	71	74

Results shown are the percentage of bone marrow-derived DCs staining positive for each marker.

DCs were untransduced or transduced with Ad2/βGal-4.

Induction of tumor-specific cytotoxic T lymphocyte response by transduced dendritic cells

The ability of DCs to induce a cytotoxic T lymphocyte (CTL) response against a melanoma-associated antigen (MAA) was evaluated *in vivo*. DCs were transduced with an Ad vector encoding human gp100 (Ad2/hugp100vl), a differentiation antigen that is expressed by most melanomas but is also present in normal melanocytes and pigmented cells of the retina. Ad2/hugp100vl-

transduced DCs (5×10^5) were administered intravenously (i.v.) to C57BL/6 mice and, 15 days later, spleens were collected for assessment of CTL activity.

Separate groups of mice were also treated with vehicle as a negative control or with the Ad2/hugp100v1 vector itself for comparison. The vector was delivered under conditions previously determined to be optimal for immunization (3×10^9 i.u., intradermally).

After *in vitro* re-stimulation with syngeneic fibroblasts transduced with Ad2/hugp100v1, effector splenocytes were tested for cytolytic activity against ^{51}Cr -labeled target fibroblasts that were either untransduced or transduced with Ad2/hugp100v1 or wild-type (WT) E3-deleted Ad (Ad2 Δ 2.9). The CTLs were also tested against B16 tumor cells, a cell line originally derived from a spontaneously arising melanoma in C57BL/6 mice which expresses the murine equivalent of human gp100.

As expected, mice treated with vehicle failed to develop any significant CTL activity against any of the targets (Figure 2A). Mice immunized with transduced DCs developed high levels of CTL activity against target fibroblasts infected with the Ad2/hugp100v1 vector. Interestingly, the bulk of the CTL response appeared to be directed against the hugp100 transgene product rather than adenoviral protein(s) since there was very little lysis of fibroblasts infected with WT Ad (Figure 2B).

Mice immunized i.d. with the Ad2/hugp100v1 vector itself, developed robust but comparatively lower levels of CTL activity against Ad2/hugp100v1-transduced fibroblasts. Furthermore, in contrast to the response obtained with transduced DCs, a significant proportion of the CTL response appeared to be specific for Ad antigen as indicated by the greater level of lysis of fibroblasts infected with WT Ad (Figure 2C). Importantly, CTLs from mice immunized with transduced DCs and, to a lesser extent, with Ad vector, were both able to lyse B16 tumor cells indicating that the CTLs raised against human gp100 also recognized the endogenous mouse gp100 expressed by the tumor cells (Figures 2B and 2C).

Immunization with Ad Vector-Transduced DCs Induces Anti-Tumor Protection

Groups of 5 C57BL/6 mice were immunized against the gp100 melanoma antigen with an intravenous injection of 5×10^5 bone marrow-derived dendritic cells (DCs) transfected with adenovirus vector encoding mouse gp100 (Ad2/mgp
5 100 DCs) or human gp100 (Ad2/hugp100 DCs). Uninfected DCs served as a negative control. Two weeks after immunization, the mice were challenged with a subcutaneous injection of 2×10^4 B16 melanoma cells and tumor growth was monitored over time. The results which are shown in Figures 1A through 1D and 3 indicate that immunization of the mice with the heterologous human gp100
10 antigen was more effective than immunization with the homologous mouse gp100 antigen in inducing protective immunity against B16 melanoma cells.

Cross-reactivity between human and murine gp100 CTL epitopes

ELISPOT analysis on spleen cells from mice immunized with
15 Ad2/hugp100-transduced DCs confirmed the presence of splenic T lymphocytes specific for a dominant CTL epitope from human gp100 (Figure 4). Importantly, the CTLs, which were raised against hugp100, showed cross-reactivity against the corresponding murine gp100 epitope. This finding is in agreement with the observation of cross-reactivity at the CTL level (Figure 2) and the resistance of
20 human gp100-immunized mice to challenge with B16 melanoma cells positive for murine gp100 (Figures 1A through 1D and 3).

As expected, spleen cells from mice immunized with Ad2/hugp100-transduced DCs did not display any significant reactivity against a known CTL epitope from ovalbumin and spleen cells from mice that received DCs transduced
25 with Ad2/empty vector did not show any significant reactivity against any of the peptides (Figure 4).

It is to be understood that while the invention has been described in conjunction with the above embodiments, that the foregoing description and the
30 following examples are intended to illustrate and not limit the scope of the invention. For example, any of the above-noted compositions and/or methods can

be combined with known therapies or compositions. Other aspects, advantages and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

CLAIMS

1. A substantially pure population of educated, antigen-specific immune effector cells produced by culturing naïve immune effector cells with antigen-presenting cells (APCs) cells which express a heterologous or an altered antigen distinct from the corresponding native self-antigen.
2. The population of claim 1, wherein the antigen presenting cells (APCs) are dendritic cells.
3. The population of claim 1, wherein the immune effector cells are cytotoxic T lymphocytes (CTLs).
4. The population of claim 1, wherein the APC have been genetically modified.
5. The population of claim 1, wherein immune effector cells have been genetically modified.
6. The population of claim 4, wherein the antigen-presenting cells comprise an exogenously added polynucleotide encoding the heterologous or altered antigen.
7. A composition comprising the population of claim 1 and a carrier.
8. The composition of claim 7, wherein the carrier is a pharmaceutically acceptable carrier.
9. The method of claim 1, wherein the self-antigen is selected from the group consisting of gp100, MART1, MUC1, HER-2, CEA, PSA, prostate

membrane specific antigen (PSMA), tyrosinase, tyrosinase related proteins 1 or 2 (TRP-1 and TRP-2), NY-ESO-1, and GA733.

5 10. A method of inducing an immune response to a native self-antigen in a subject, comprising administering to the subject an effective amount of a heterologous or altered antigen corresponding to the native self-antigen and under the conditions that induce an immune response to the native self-antigen.

10 11. The method of claim 10, further comprising administering an effective amount of a cytokine to the subject.

 12. The method of claim 10, further comprising administering an effective amount of a co-stimulatory molecule to the subject.

15 13. The method of claim 10, wherein more than one heterologous or altered antigen that induces an immune response to the native or self-antigen.

 14. The method of claim 10, wherein the native self-antigen is a tumor antigen.

20 15. The method of claim 14, wherein the tumor antigen is selected from the group consisting of gp100, MART1, MUC1, HER-2, CEA, PSA, prostate membrane specific antigen (PSMA), tyrosinase, tyrosinase related proteins 1 or 2 (TRP-1 and TRP-2), NY-ESO-1, and GA733.

25 16. A method of inducing an immune response to a native self-antigen in a subject, comprising administering to the subject an effective amount of an antigen-presenting cell expressing a heterologous or altered antigen corresponding to the native self-antigen and under conditions that induce an immune response to the self-antigen in the subject.

30

17. The method of claim 16, further comprising administering an effective amount of a cytokine to the subject.

18. The method of claim 16, further comprising administering an effective amount of a co-stimulatory molecule to the subject.

19. The method of claim 16, wherein the antigen is a tumor antigen.

20. The method of claim 16, wherein the antigen-presenting cell is a dendritic cell.

21. The method of claim 20, wherein the self-antigen is selected from the group consisting of gp100, MART1, MUC1, HER-2, CEA, PSA, prostate membrane specific antigen (PSMA), tyrosinase, tyrosinase related proteins 1 or 2 (TRP-1 and TRP-2), NY-ESO-1, and GA733.

22. The method of claim 16, further comprising administering more than one heterologous or altered antigen that induces an immune response to the native or self-antigen.

23. The method of claim 16, wherein the antigen-presenting cell is genetically modified.

24. The method of claim 23, wherein the genetically modified cell expresses a heterologous or altered antigen corresponding to the native self-antigen.

25. The method of claim 16, further comprising genetically modifying the APC to express a cytokine.

26. The method of claim 16, further comprising genetically modifying the APC to express a co-stimulatory molecule.

5 27. A method of adoptive immunotherapy, comprising administering to a subject an effective amount of a population of educated, antigen-specific immune effector cells of claim 1.

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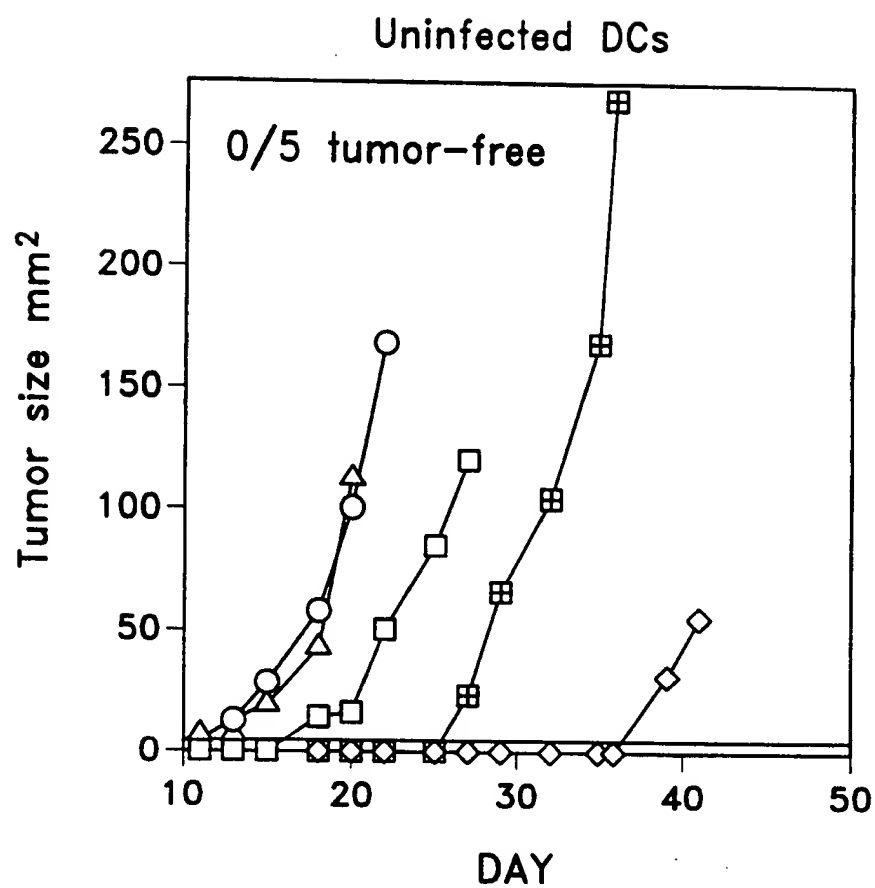


FIG. 1A

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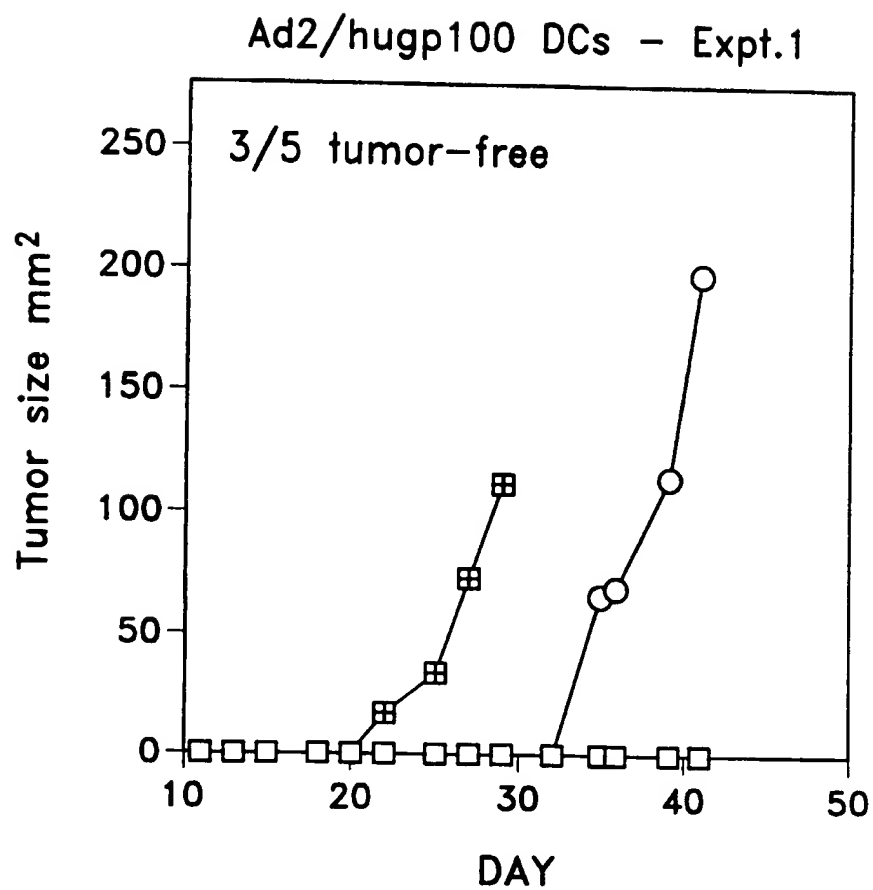


FIG. 1B

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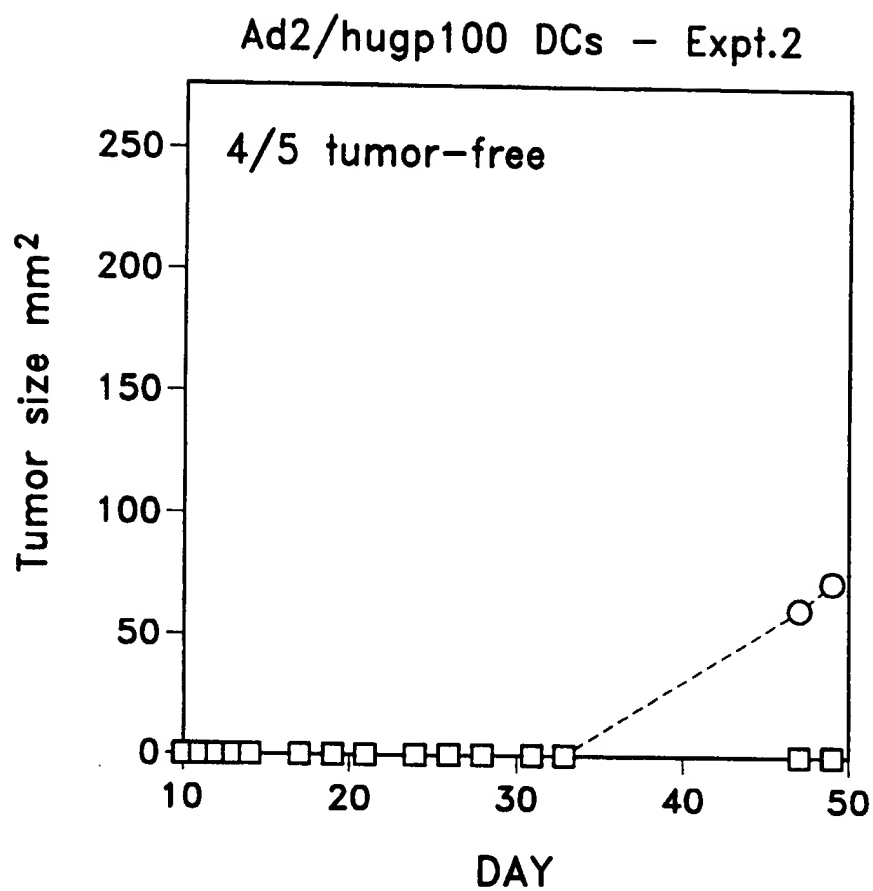


FIG. 1C

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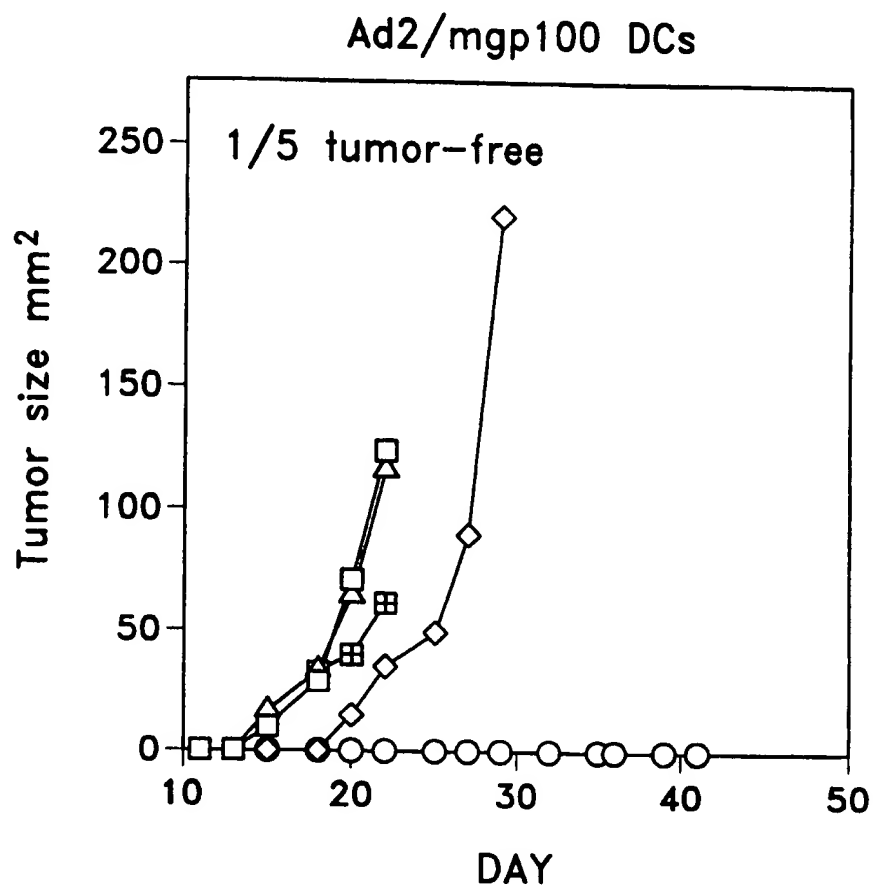


FIG. 1D

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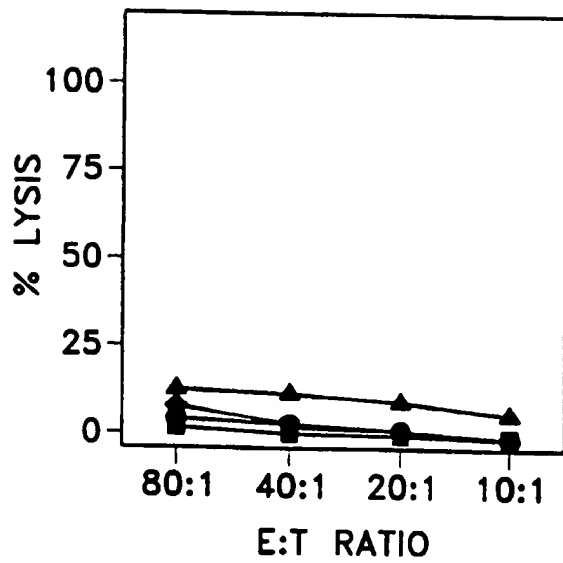


FIG. 2A

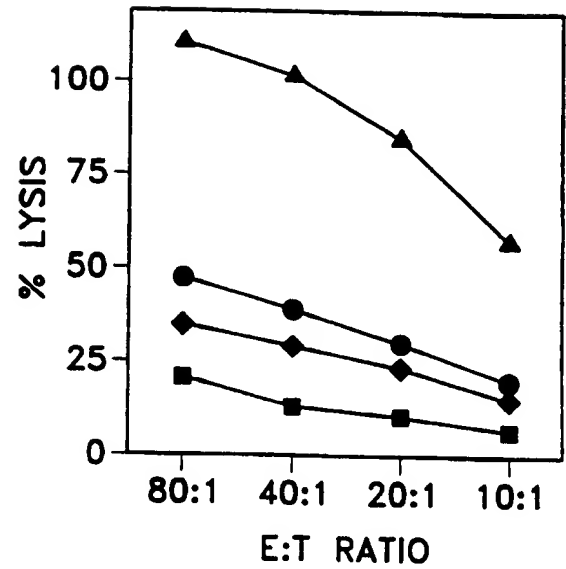


FIG. 2B

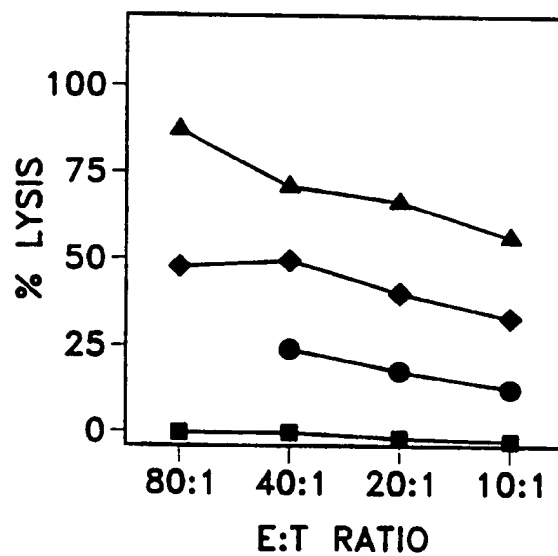


FIG. 2C

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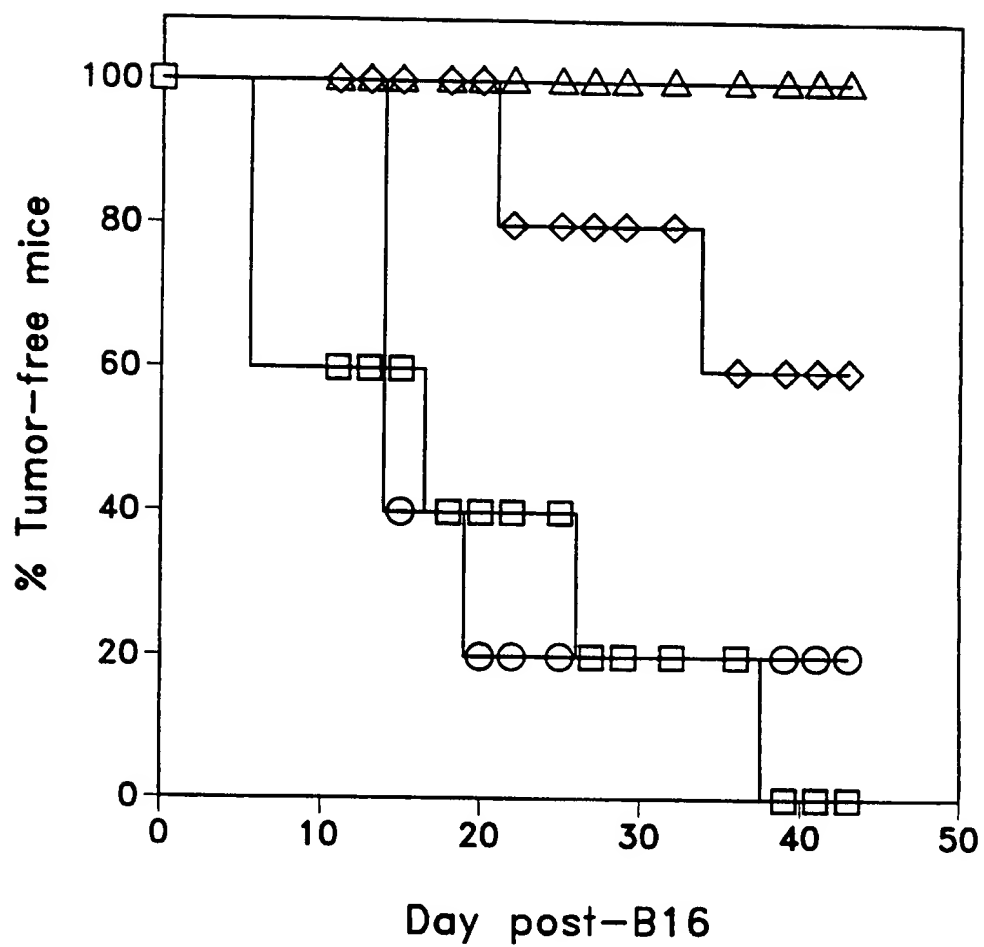


FIG. 3

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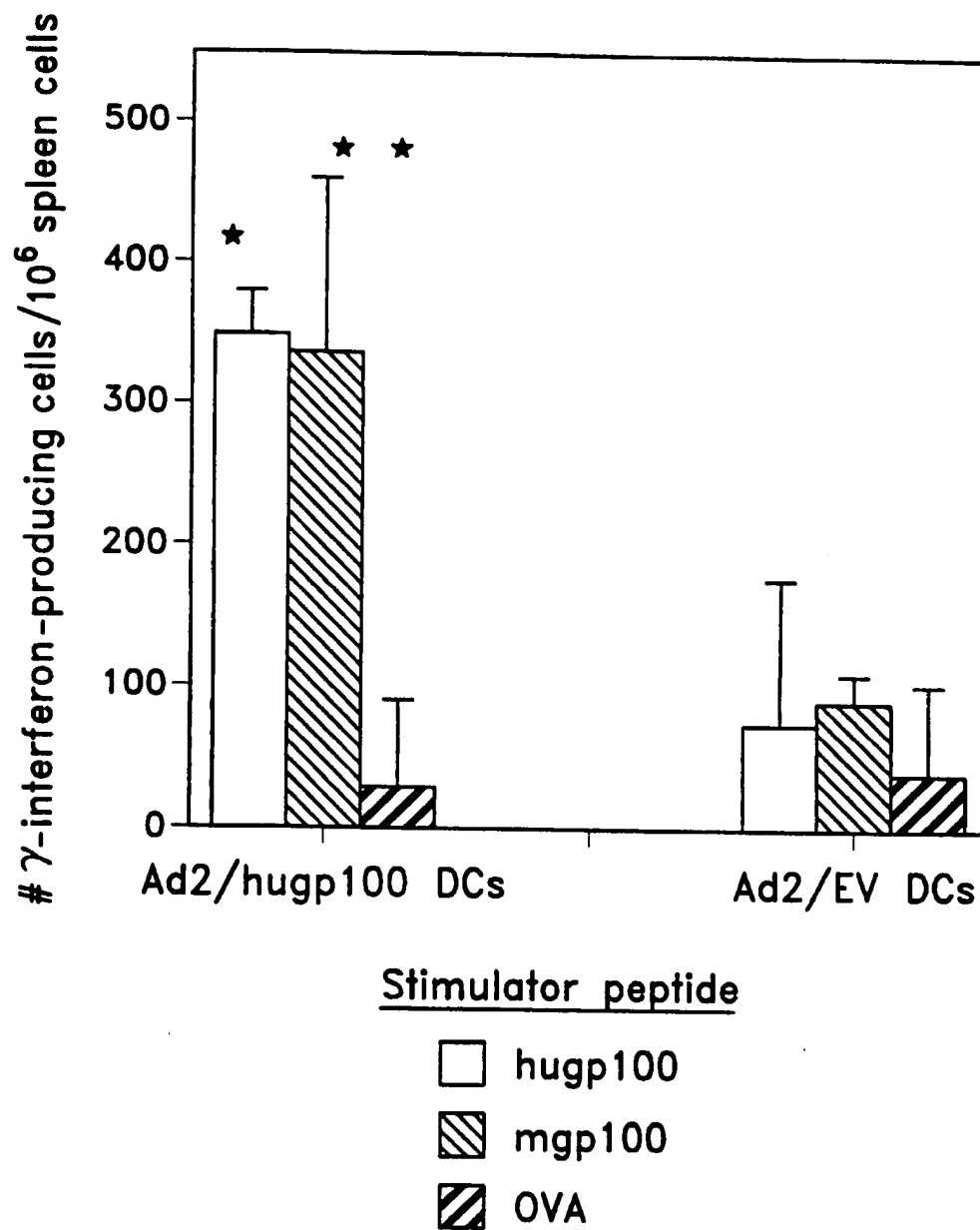


FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/06039

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A01N 63/00; A61K 38/02, 39/00; C12N 5/06, 5/10

US CL : 424/184.1, 277.1, 93.7, 435/325, 514/2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1, 277.1, 93.7, 435/325, 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG medicine index, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MOIOLA, L. et al. T-helper epitopes on human nicotinic acetylcholine receptor in myasthenia gravis. Ann. NY Acad. Sci. 1993, Vol. 681, pages 198-218, see entire document, especially pages 199 and 214.	1-4 6-8, 10
X	PACHNER, A.R. et al. An immunodominant site of acetylcholine receptor in experimental myasthenia mapped with T lymphocyte clones and synthetic peptides. Immunol. Lett. February 1989, Vol. 20, No. 3, pages 199-204, see entire document.	1-8, 10, 27

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

09 JUNE 1999

Date of mailing of the international search report

13 AUG 1999

 Name and mailing address of the ISA/US
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 Box PCT
 Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

F. PIERRE VANDERVEGT

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06039

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BELLONE, M. et al. Experimental myasthenia gravis in congenic mice. Sequence mapping and H-2 restriction of T helper epitopes on the alpha subunits of Torpedo californica and murine acetylcholine receptors. Eur. J. Immunol., 1991, Vol. 21, pages 2303-2310, see entire document..	1-4, 6-8 10, 12
X	INFANTE, A. J. et al. Determinant selection in murine experimental autoimmune myasthenia gravis. Effect of the bm12 mutation on T cell recognition of acetylcholine receptor epitopes. J. Immunol. 01 May 1991, Vol. 146, No. 9, pages 2977-2982, see entire document.	1-8, 10
Y	CHAKRABORTY, M. et al. Preclinical evaluation in nonhuman primates of an anti-idiotypic antibody that mimics the carcinoembryonic antigen. J. Immunotherapy. February 1995, Vol. 18, No. 2, pages 95-103, see entire document, especially abstract.	1-4, 6-8, 10-12, 14-15, 19, 21, 24
X - Y	US 5,648,219 A (MacKAY et al) 15 July 1997, see entire document.	16-18, 20, 22, 23, 25, 26 ----- 19, 21, 24
X	XU, Q. et al. An immunogenic self-peptide for T cells in mice with experimental myasthenia. NY Acad. Sci. 1993, Vol. 681, pages 1-4, see entire document.	1-8, 10, 12
X	PACHNER, A.R. et al. Suppressor T-cell lines and hybridomas in murine myasthenia. NY Acad. Sci. 1986, Vol. 505, pages 619-627, especially page 622.	1-8, 10, 12, 27

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06039

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 9, 13
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claim is drawn to "The method of claim 1," but claim 1 is a product, not a method. Claim 13 is incomplete.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.